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CONTENTS OF THIS ISSUE

The Use of Chloromethylation Products in Preparation of Substances of Pharmacologic Interest 115
George P. Hager and Wei-Chin Liu

Comparative Toxicities of Quercetin and Quercitrin 119
Anthony M. Ambrose, Dorothy J. Robbins, and Floyd DeEds

Spray-Drying of Insoluble Weakly Acidic Pharmaceuticals Through Their Ammonium Salts 122
T. Higuchi, M. Gupta, and L. W. Busse

Determination of Certain Properties of Ointment Bases by Use of Chick Embryos 125
Matthew C. Hunter and F. Jane Smith

A Cylinder Dispenser for Use in Agar Diffusion Assays 131
E. H. Christiansen and W. A. Zygmunt

Use of Bromsulphalein for Assay of Cobra Venom 133
George S. Warner, John H. Brewer, and H. A. Brown Dunning, Jr.

An Investigation of the Chronic Toxicity and Acceptability of Castrix 135
James M. Crampton and Elbert Voss

Gravimetric Determination of Histamines 138
Morton Schmall, Ernest G. Wollish, and Julius Galender

Studies of Lactic Acid Producing Organisms in a Microbiological Assay of Penicillin 141
Muriel C. Vincent, Edward Krupski, and Louis Fischer

Optical Crystallographic Properties of Organic Compounds. III. Esters of *p*-Hydroxybenzoic Acid 143
Raymond N. Castle

Digitalis Extraction Studies. III 146
Raymond E. Hopponen and Ole Gisvold

Digitalis Extraction Studies. IV 152
G. G. Krishnamurty and Ole Gisvold

Some Derivatives of *o*-Thymotinic Acid 155
Shao-Chia Chou, Paul J. Jannke, Joseph B. Burt, and LaVerne D. Small

A Comparative Study of Tablet Disintegrating Agents 157
Herbert Micheal Gross and Charles H. Becker

Pharmacological Actions of *Paeonia officinalis* 162
Duane G. Wenzel and Albert R. Haskell

A Comparison of the Effects of Anthallan, Pyribenzamine, and Epinephrine on Smooth Muscle 164
Rebecca C. Hellerman and Lloyd W. Hazleton

Book Reviews 167

Interim Revision Announcement, Pharmacopeia of the United States Fourteenth Revision 170

Advertising ..... Cover pages 2, 3, 4, and pp. I-VIII

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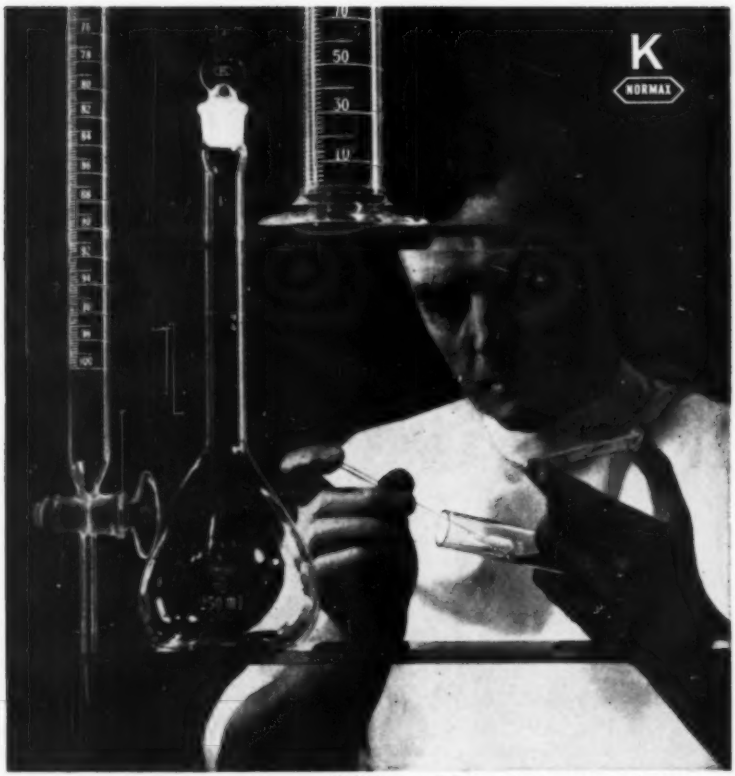

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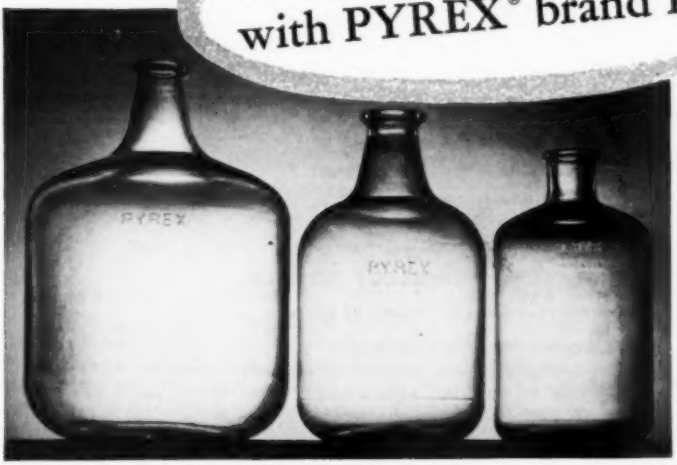

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The Use of Chloromethylation Products in Preparation of Substances of Pharmacologic Interest*

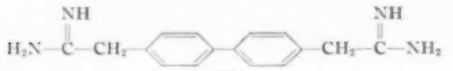
By GEORGE P. HAGER and WEI-CHIN LIU†,‡

Several diamidines having a homologous relationship to various chemotherapeutic diamidines have been prepared from the bis(chloromethylation)-derivatives of naphthalene, biphenyl, and bibenzyl. Two of these, 4,4′-bis(amidinomethyl)bibenzyl dihydrochloride and 4,4′-bis(amidinomethyl)biphenyl dihydrochloride, were found to possess a high order of antibacterial activity when tested *in vitro* using cultures of *Mycobacterium tuberculosis*, H37Rv. The ease of reaction of the intermediate chloromethyl derivatives with various nucleophilic reagents makes possible their conversion to a number of substances of possible pharmacologic interest. The preparation of basic ethers by reaction of the chloromethyl derivatives with sodium diethylaminoethoxide is described.

THE AVAILABILITY of a wide variety of chloromethyl derivatives of aromatic hydrocarbons and related compounds (1) and the ease of their reactions with nucleophilic reagents suggested the possibility that such chloromethylation products might be useful starting materials for preparation of compounds of pharmacologic interest. The present study has dealt with the bis(chloromethylation)-derivatives of polynuclear aromatic hydrocarbons, *viz.*, naphthalene, biphenyl, and bibenzyl, because these could be used as intermediates in the preparation of amidines which in a sense have a homologous relationship to various diamidines with chemotherapeutic activity. A further consideration in this choice of bis(chloromethyl)naphthalene, etc., as desirable intermediates, was the possibility of their conversion to polyfunctional analogs of various other pharmacodynamically active substances possessing a single functional group.

The steric orientation of amidine groups in two of the diamidines prepared in this study, 4,4′-bis(amidinomethyl)biphenyl and 4,4′-bis(amidinomethyl)bibenzyl, conforms to the pattern which is considered optimum for chemotherapeutic activity, in that the amidine groups are separated by intramolecular distances not less than that found in stilbamidine nor greater than that found in pentamidine (2). The biphenyl derivative (I) has a structural-isomeric relationship to dihydrostilbamidine, while the bibenzyl derivative (II) has the form of a bis(homo)dihydrostilbamidine.

* Received August 31, 1951, from the School of Pharmacy, University of Maryland, Baltimore.
Presented to the Scientific Section, A. Ph. A., Buffalo meeting, August, 1951.
† Bristol Laboratories Research Fellow, 1949–1951.
‡ The authors wish to acknowledge with appreciation the support given this work by the Bristol Laboratories, Inc.



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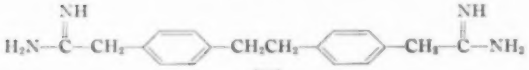
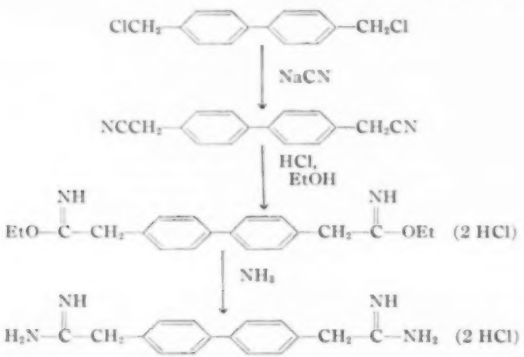


(II)

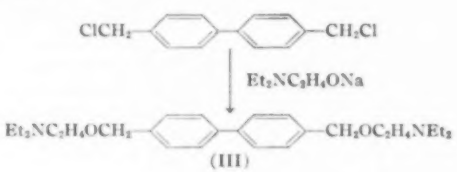
Because of the successful use of stilbamidine in treatment of kala-azar complicated by pulmonary tuberculosis (3), interest in the antibacterial activity of the amidines prepared in this investigation, on *Mycobacterium tuberculosis*, was aroused, and *in vitro* and preliminary *in vivo* antibacterial studies were carried out. Compound II was also included in a study of the effects of amidines on the interfacial denaturation of protein (4) in which it was found to inhibit completely the denaturation of albumin and ribonuclease at oil-water interfaces, whereas stilbamidine and propamidine enhanced the denaturation.

The amidines were prepared from the chloromethyl intermediates by way of the corresponding nitriles and imido esters as indicated by the following equation:



Considerable difficulty was encountered in obtaining the amidine hydrochlorides in pure condition, probably because of a tenacious retention of ammonium chloride by the product.

Basic ethers were prepared by substitution of the chloromethyl intermediates by reaction with sodium diethylaminoethoxide.



(III)

By means of the reaction indicated, 4,4′-bis(2-diethylaminoethoxymethyl)biphenyl (III) and 4,4′ - bis(2 - diethylaminoethoxymethyl)bibenzyl were prepared and isolated as the corresponding dihydrochlorides. Like the antihistaminic agent, diphenhydramine, these compounds are aralkyl ethers of basic alcohols. Of possible interest also is their relationship to a number of basic ethers of phenols in the biphenyl, bibenzyl, and stilbene series which have been shown to possess interesting pharmacodynamic properties, e. g., coronary dilating and antifibrillatory activity of 2(2-diethylaminoethoxy)biphenyl (5), 4(2-diethylaminoethoxy)bibenzyl (6), and 4(2-diethylaminoethoxy)-*alpha,alpha′*-dimethylstilbene (7). Efforts to prepare similar ethers from 1,5-bis(chloromethyl)naphthalene were unavailing. The high-melting nitrogen-free and halogen-free product invariably obtained is suggestive of the possibility that an intermolecular condensation polymerization which was not observed in preparation of the other ethers or a very interesting intramolecular condensation might have taken place.

EXPERIMENTAL[1]

4,4′-Bis(chloromethyl)bibenzyl.—This compound was prepared by the method described by Reichstein and Oppenauer (8) with some modifications.

Bibenzyl (100 Gm., 0.55 mole) was mixed with 300 Gm. (2.6 moles) of dichloromethyl ether and 42 Gm. (0.3 mole) of freshly fused and finely powdered zinc chloride. The mixture was stirred mechanically for ten hours at 35°, poured into 1 L. of ice water and extracted with ether. After washing the ether extract with water and 10% sodium carbonate and drying with anhydrous sodium sulfate, the ether was removed completely by distillation. The residual oil was distilled under reduced pressure and the fraction collected at 170–215° (0.2 mm.) was recrystallized from petroleum ether. A yield of 35 Gm. (22.8% of theoretical) of a white crystalline solid, m. p. 96–97°, reported m. p. 97° (8), was obtained.

4,4′-Bis(chloromethyl)biphenyl.—The preparation of this compound was accomplished by treatment of biphenyl with trioxymethylene, fused zinc chloride, and dry hydrogen chloride, as directed by Braun (9).

1,5-Bis(chloromethyl)naphthalene.—A suspension of 90 Gm. (0.7 mole) of naphthalene in a mixture of 300 Gm. of 35–40% aqueous solution of formaldehyde and 200 Gm. (2.1 moles) of concentrated hydrochloric acid was treated with a rapid stream of dry hydrogen chloride for twelve hours while refluxing and stirring. The solid material which separated on cooling was washed with water and extracted with 200 cc. of boiling acetone. A crystalline precipitate from the acetone extract was recrystallized from 95% ethyl alcohol, washed with ether, and recrystallized again from alcohol. A yield of 55 Gm. (35% of theoretical) of 1,5-bis(chloromethyl)naphthalene, m. p. 171–172°, reported m. p. 171.5 to 172.5° (10), was obtained.

4,4′-Bis(cyanomethyl)bibenzyl.—A 71% yield of this compound was obtained by substitution of 4,4′-bis(chloromethyl)bibenzyl using sodium cyanide in a mixture of alcohol and water according to the directions of Reichstein and Oppenauer (8).

4,4′-Bis(cyanomethyl)biphenyl.—The crude product obtained by reaction of 4, 4′-bis(chloromethyl)-biphenyl with sodium cyanide in dilute alcohol and removal of solvent by distillation under reduced pressure was washed with water and recrystallized from alcohol. A 62% yield of yellow crystalline material, m. p. 184°, reported m. p. 184° (9), was obtained.

1,5-Bis(cyanomethyl)naphthalene.—The product of the reaction of 1,5-bis(chloromethyl)naphthalene with sodium cyanide, obtained in the usual manner, was recrystallized from acetone. A 90% yield of 1,5-bis(cyanomethyl)naphthalene, light yellow crystals, m. p. 188–189°, reported m. p. 189° (11), was obtained.

4,4′-Bis(amidinomethyl)bibenzyl Dihydrochloride.—Dry hydrogen chloride was passed into a solution of 25 Gm. (0.096 mole) of 4,4′-bis(cyanomethyl)bibenzyl in 13 cc. of absolute ethyl alcohol and 30 cc. of Skellysolve-B contained in a 1-L. tared filtration flask immersed in an ice-salt mixture, until an increase in weight of 8.8 Gm. (corresponding to 0.24 mole of hydrogen chloride) resulted. The mixture was transferred to a pressure bottle, stoppered tightly and let stand at 0° for seven days, during which time the crystalline imido ester hydrochloride precipitated.

The imido ester hydrochloride was filtered from the reaction mixture, ground to a paste with absolute ethyl alcohol, and added to an excess of a 13% solution of ammonia in alcohol. The mixture was stirred for four hours while the imido ester hydrochloride dissolved and ammonium chloride precipitated. After filtering the mixture with suction, the filtrate was evaporated to about one-half of its original volume under reduced pressure. The residual solution was decolorized with charcoal and treated while warm with anhydrous ether until it became cloudy. A fine crystalline precipitate separated on cooling and was recrystallized repeatedly from mixtures of absolute ethyl alcohol and anhydrous ether. A yield of 15 Gm. (42.5% of theoretical) of 4,4′-bis(amidinomethyl)bibenzyl dihydrochloride, white crystals, m. p. 281°, was obtained.

Anal.—Calcd. for $C_{18}H_{24}Cl_2N_4$: N, 15.25. Found: N, 14.96, 14.94.

4,4′-Bis(amidinomethyl)biphenyl Dihydrochloride.—4,4′-Bis(cyanomethyl)biphenyl was treated with hydrogen chloride in alcohol and the resulting imido ester hydrochloride was treated with ammonia in alcohol by the same procedure employed for preparation of 4,4′-bis(amidinomethyl)bibenzyl dihydrochloride. A 38% yield of a white crystalline product, m. p. 288° dec., was obtained.

This compound was included in a series of diamidines reported by Ashley and others (12), but no physical constants were given.

Variable and somewhat high nitrogen values in the analytical data obtained for several samples, prepared by repetition of the above procedure, indicated presence of ammonium chloride which could not be removed by the purification measures employed.

1,5-Bis(amidinomethyl)naphthalene Dihydrochloride.—Application of the above procedure for conversion of 1,5-bis(chloromethyl)naphthalene by way of the imido ester hydrochloride to 1,5-bis(amidinomethyl)naphthalene dihydrochloride, resulted in the formation of a 26% yield of a white crystalline solid, decomposing at 269–271°. The same discrepancies were encountered in analytical data obtained for several samples prepared in this way that were mentioned in connection with 4,4′-bis(amidinomethyl)biphenyl dihydrochloride.

4,4′ - Bis(2 - diethylaminoethoxymethyl)bibenzyl Dihydrochloride.—A solution of 0.55 Gm. (0.024 equivalent) of sodium in 10 cc. of 2-diethylamino ethanol was prepared and the excess 2-diethylaminoethanol was removed by distillation under reduced pressure. The solid residue was taken up in 20 cc. of dry xylene and treated with 2.3 Gm. (0.008 mole) of 4,4′-bis(chloromethyl)bibenzyl and refluxed gently for five hours.

The reaction mixture was shaken with a small amount of 20% sodium hydroxide and extracted with ether. The ether solution of the free basic ether was extracted with 10% hydrochloric acid. The aqueous solution of the hydrochloride of the basic ether obtained in this way was made alkaline

[1] All melting points are uncorrected and were observed by use of a Fisher-Johns melting point apparatus.

with 10% sodium hydroxide, and the basic ether extracted with ether. After drying the ether solution of the basic ether with anhydrous sodium sulfate, the ether was distilled and the residue heated at 85° under reduced pressure for a short time to remove any 2-diethylaminoethanol. The basic ether, obtained as a yellow oil, was converted to the hydrochloride in the usual way, and this was purified by recrystallization from mixtures of alcohol and ether. A yield of 2.2 Gm. (52% of theoretical) of 4,4′-bis-(2-diethylaminoethoxymethyl)bibenzyl dihydrochloride, white crystals, m. p. 124–125°, was obtained.

Anal.—Calcd. for $C_{28}H_{46}Cl_2N_2O_2$: N, 5.46. Found: N, 5.39, 5.60.

4,4′-Bis(2-diethylaminoethoxymethyl)biphenyl Dihydrochloride.—4,4′-Bis(chloromethyl)biphenyl was treated with sodium 2-diethylaminoethoxide in xylene, and the product purified as described in the preparation of the bibenzyl analog.

A 64% yield of 4,4′-bis(2-diethylaminoethoxymethyl)biphenyl dihydrochloride, white crystals, m. p. 185–186°, was obtained.

Anal.—Calcd. for $C_{26}H_{42}Cl_2N_2O_2$: N, 5.78. Found: N, 5.73, 5.60.

BACTERIOLOGY

Preliminary studies[2] of the antibacterial activity of the amidines in cultures of *Mycobacterium tuberculosis*, H37Rv justified a more extensive study of their tuberculostatic activity. The results of the latter tests,[3] along with toxicity data, are tabulated in Table I.

Since the lowest concentration of streptomycin inhibiting growth of *Mycobacterium tuberculosis*, H37Rv under conditions of this test was generally 0.0004 to 0.0008 mg./cc., it was concluded that the bibenzyl derivative is about one-fifth, and the biphenyl derivative about one-tenth, as active *in vitro* as streptomycin.

SUMMARY

1. Several diamidines, 4,4′-bis(amidinomethyl)bibenzyl, 4,4′-bis(amidinomethyl)biphenyl, and 1,5-bis(amidinomethyl)naphthalene, have been prepared by way of intermediates obtained by chloromethylation of the corresponding hydrocarbons. Because of an apparently tenacious retention of ammonium chloride, satisfactory analytical data were not obtained for the biphenyl and naphthalene derivatives.

2. Substitution of the chloromethyl intermediates using sodium 2-diethylaminoethoxide resulted in formation of 4,4′-bis(2-diethylaminoethoxymethyl)bibenzyl dihydrochloride and 4,4′-bis(2-diethylaminoethoxymethyl)biphenyl dihydrochloride. A basic ether could not be obtained in this way from 1,5-bis(chloromethyl)-naphthalene.

3. The amidine derivatives of biphenyl and bibenzyl were found to have a high order of antibacterial activity in cultures of *Mycobacterium tuberculosis*, H37Rv.

Table I.—Antibacterial Activity and Toxicity of Amidines

Compound	Lowest Inhibiting Concentration,[a] Mg./Cc.	LD_{50},[b] Mg./Kg.
4,4′-bis(amidinomethyl)-bibenzyl dihydrochloride	0.002–0.004	64.5
4,4′-bis(amidinomethyl)-biphenyl dihydrochloride[c]	0.004–0.008	26.7
1,5-bis(amidinomethyl)-naphthalene dihydrochloride[c]	0.14	43.2

[a] Lowest concentration (mg./cc.) effective in inhibiting growth of *Mycobacterium tuberculosis*, H37Rv.
[b] LD_{50} in mg./Kg. of body weight, administered intraperitoneally to white mice.
[c] Sample contaminated with a small amount of ammonium chloride.

[1] Preliminary studies of the antibacterial activity of the amidines were carried out by Dr. Donald E. Shay, and the authors acknowledge with thanks his most helpful cooperation in this investigation.

[2] The authors are indebted to Bristol Laboratories, Inc., for the bacteriological and toxicity data herein reported.

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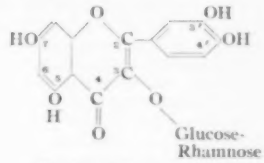
Comparative Toxicities of Quercetin and Quercitrin*

By ANTHONY M. AMBROSE, DOROTHY J. ROBBINS, and FLOYD DeEDS

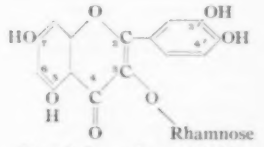
Data are given on the toxicity of quercetin and quercitrin after intravenous administration in rabbits and rats after oral ingestion of diets containing as much as 1 per cent of quercetin or quercitrin for approximately 410 days. The results indicate a low order of toxicity as judged by growth, amount of food consumed, hematological findings, organ weights, and microscopical examination of stained section of visceral organs.

AT THE PRESENT time rutin is being used extensively for therapeutic purposes, alone and in a variety of pharmaceutical formulations. The safety of using rutin over long periods of time and the need for investigating its safety have been reported by Wilson, Mortarotti, and Doxtader (1). One of the questions arising in the course of our investigations on the acute and chronic toxicity and mechanism of action of rutin is concerned with the role, if any, played by the glucose and rhamnose attached to the number 3 carbon of quercetin. Evidence has been presented from this laboratory (2–5) to the effect that quercetin and quercitrin exert the same physiological effects as rutin. The present studies were undertaken to determine whether the absence of the sugars as in quercetin, or the presence of rhamnose alone as in quercitrin, would result in greater chronic toxicity as compared with rutin.

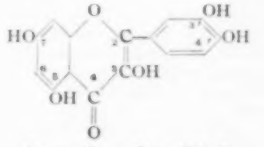
Quercetin (meletin, flavin, sophoretin), a tetrahydroxy flavonol present in the rind of many fruits, is a yellow crystalline powder with a melting point variously reported of 309–314°. It is almost insoluble in water, but soluble in alkalies and in alcohol. Quercitrin (quercimelin, quercetin rhamnoside, quercitrinic acid), the tetrahydroxy rhamnoside present in the bark of *Quercus tinctoria* Bart., *Fagaceae*, and other plants, is a yellow crystalline powder, melting at 182–185°, and having the same solubility characteristics as quercetin. The chemical relationship of rutin, quercitrin, and quercetin is shown in the accompanying formulas. Since there is every reason to believe that the physiological action of the flavonol glycosides is due to the flavonol moiety rather than to the glycosides *per se*, quercetin and quercitrin should have equal or greater therapeutic value on a weight basis, as compared with rutin.



Rutin, mol. wt. 610.51



Quercitrin, mol. wt. 448.17



Quercetin, mol. wt. 302.09

EXPERIMENTAL

Acute Toxicity.—The acute toxicity of quercetin and quercitrin was determined in rabbits by intravenous injections. Quercetin was solubilized with methyl glucamine (kindly supplied by the Abbott Laboratories) as follows: For each gram of quercetin 2 Gm. of methyl glucamine was added in 10 ml. of water. Quercitrin was solubilized by adding 1 Gm. of quercitrin to 5 ml. of propylene glycol.

In four rabbits receiving 100 mg./Kg. of solubilized quercetin intravenously no toxic reactions were observed!

In 14 rabbits receiving single doses of 100–150 mg./Kg. of quercitrin intravenously, no symptoms were observed that could be ascribed to quercitrin. In six rabbits receiving two injections each (one hour apart) of 100, 120, and 136 mg./Kg., no toxic symptoms were apparent.

In all of the rabbits receiving either quercetin or quercitrin, the urine voided naturally or by catheterization was highly pigmented, staining paper yellow, and intensification in color of the urine with sodium hydroxide suggested rapid elimination of both quercetin and quercitrin.

Chronic Toxicity.—Experiments were carried out on weanling albino rats weighing 45–50 Gm. each. Both sexes were equally represented in control and experimental groups. The rats were divided into groups of five of each sex and placed on one of the following diets: basic diet, 0.25, 0.5, and 1% quercetin or quercitrin. The basic diet on which the control group of rats was placed and to which quer-

* Received September 4, 1951, from the Pharmacology Laboratory, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture, Albany 6, Calif.

cetin[1] or quercitrin[1] was added had the following percentage composition: casein 10, corn meal 73, linseed oil meal 10, alfalfa 2, bone ash 1.5, sodium chloride 0.5, and cod liver oil 3. Free access to food and water was permitted at all times. At weekly intervals the weights of individual rats in each group and the food consumption were recorded. All rats were kept on the respective diets for approximately 410 days before they were autopsied. Growth curves for both male and female rats on the respective diets are given in Figs. 1 and 2. From these curves it can readily be seen that growth on all levels of quercetin or quercitrin was essentially the same as that for the control rats on the basic diet alone. Hematological studies consisting of hemoglobin determination and red and white cell counts were made after approximately 70, 280, and 400 days on the respective diets. No significant difference was found between the rats on the experimental diet and those on the basic diet alone in any one of the three periods tested.

On completion of the feeding experiments all surviving rats were killed with ether, exsanguinated, and the adrenals, kidneys, spleen, liver, heart, and in the case of males, the testes, were weighed. The mean organ weights and statistical treatment of the data are summarized in Table I. No statistically significant difference was found between the organ weights of rats on the experimental diets and those on the control diet.

In addition to the above tissues, thyroid, lungs, pancreas, stomach, small intestines, and bladder from all rats, and reproductive organs of the females were fixed in 4% formaldehyde solution. Paraffin sections stained with hematoxylin-eosin were prepared. Histopathological study of the sections was made by Dr. L. J. Rather, Department of Pathology, Stanford University School of Medicine, San Francisco, Calif., who reported no evidence of tissue damage that could be attributed to diets fed.

DISCUSSION

The amounts of quercetin and quercitrin administered in the daily diets of the experimental animals were far in excess of the quantities of these agents that might be used therapeutically. In comparison with the earlier report (1) on the chronic toxicity of rutin it is evident that the animals on a diet containing 1.0% quercetin received twice as much of the aglycone moiety as did those on a diet containing 1.0% rutin, thus emphasizing the low order of toxicity of quercetin. The average amount of quercetin or quercitrin ingested daily over a period of 400 days for male and female rats was well over 400 mg./Kg., which would be equivalent to approximately 28 Gm. per day for a 70-Kg. man. However, during the first 35 days (susceptible period) of the feeding experiments, the average daily intake was well over 1,000 mg./Kg. on the diets containing 1% quercetin or quercitrin. For other dietary levels of quercetin or quercitrin the average daily intake, during the same period, was proportionately lower. In the light of negative histological findings and the conditions of the ex-

TABLE I.—ORGAN WEIGHTS OF RATS SURVIVING FOR APPROXIMATELY 410 DAYS ON DIETS CONTAINING QUERCETIN AND QUERCITRIN

Compound in Diet		No. of Rats	Liver, Gm. Mean Wt. ± S. E.[a]	t	Spleen, Gm. Mean Wt. ± S. E.	t	Testes, Gm. Mean Wt. ± S. E.	t	Adrenals, Gm. Mean Wt. ± S. E.	t	Kidneys, Gm. Mean Wt. ± S. E.	t	Heart, Gm. Mean Wt. ± S. E.	t
Males														
Quercetin	0.00	4	12.95 ± 0.84	..	0.623 ± 0.019	..	2.27 ± 0.46	..	0.038 ± 0.002	..	2.51 ± 0.11	..	1.31 ± 0.64	..
	0.25	4	11.45 ± 0.14	1.75	0.647 ± 0.021	0.85	3.07 ± 0.16	1.66	0.038 ± 0.002	0.03	2.18 ± 0.10	2.16	1.34 ± 0.17	0.18
	0.50	5	11.65 ± 0.43	1.36	0.630 ± 0.039	0.16	3.12 ± 0.18	1.74	0.036 ± 0.007	1.03	2.24 ± 0.06	2.13	1.11 ± 0.05	2.59
	1.00	4	10.95 ± 0.60	1.92	0.675 ± 0.036	1.28	3.57 ± 0.13	2.74	0.036 ± 0.006	1.06	2.29 ± 0.07	1.68	1.23 ± 0.03	1.19
Quercitrin	0.25	4	12.56 ± 0.36	0.40	0.595 ± 0.032	0.75	2.05 ± 0.19	0.46	0.035 ± 0.002	0.08	2.19 ± 0.03	2.68	1.48 ± 0.25	0.67
	0.50	4	10.22 ± 0.31	3.02	0.577 ± 0.045	0.94	1.43 ± 0.11	1.79	0.033 ± 0.003	0.01	2.22 ± 0.07	2.17	1.24 ± 0.11	0.53
	1.00	4	11.71 ± 0.63	1.17	0.580 ± 0.021	1.52	1.52 ± 0.19	1.53	0.037 ± 0.001	0.03	2.32 ± 0.07	1.40	1.12 ± 0.03	2.79
Females														
Quercetin	0.00	4	8.53 ± 0.44	..	0.525 ± 0.015	..	....	..	0.047 ± 0.002	..	1.57 ± 0.03	..	0.95 ± 0.05	..
	0.25	4	8.16 ± 0.74	0.43	0.467 ± 0.032	1.64	....	..	0.038 ± 0.005	1.51	1.53 ± 0.05	0.72	0.89 ± 0.04	0.80
	0.50	5	8.18 ± 0.38	0.60	0.508 ± 0.019	0.70	....	..	0.039 ± 0.002	2.84	1.54 ± 0.06	0.57	0.80 ± 0.02	2.47
	1.00	3	8.35 ± 0.38	0.31	0.445 ± 0.062	1.25	....	..	0.044 ± 0.002	0.90	1.53 ± 0.11	0.38	0.86 ± 0.06	1.08
Quercitrin	0.25	3	8.56 ± 0.59	0.04	0.477 ± 0.008	2.88	....	..	0.034 ± 0.006	1.88	1.56 ± 0.02	1.71	0.91 ± 0.02	0.75
	0.50	4	7.87 ± 0.43	1.06	0.434 ± 0.031	2.64	....	..	0.040 ± 0.001	3.01	1.58 ± 0.04	0.10	0.87 ± 0.02	1.46
	1.00	4	10.22 ± 0.51	2.50	0.468 ± 0.018	2.43	....	..	0.038 ± 0.002	3.38	1.57 ± 0.06	0.02	0.86 ± 0.04	1.39

[a] S. E. = Standard error of the mean.

[b] $t = \frac{M_1 - M_2}{\sqrt{(S.E._1)^2 + (S.E._2)^2}}$ where M_1 is the mean of the control set of observations and M_2 the mean of any other set, S. E.$_1$ and S. E.$_2$ are the standard errors of M_1 and M_2, respectively. Values of 3 or greater are considered significant.

[1] Prepared in this laboratory from orange and lemon flavin, respectively. Both were at least 97% pure. See THIS JOURNAL, 40,384(1951).

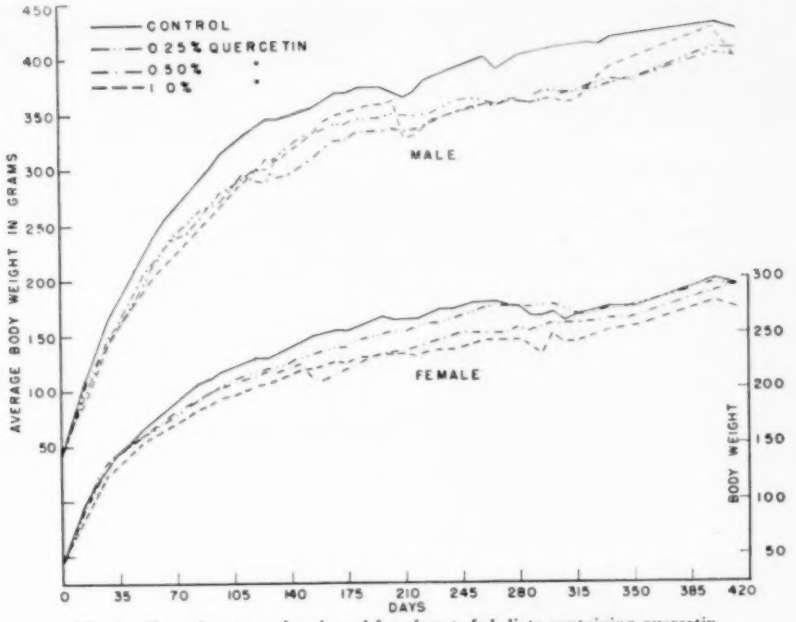


Fig. 1.—Growth curves of male and female rats fed diets containing quercetin.

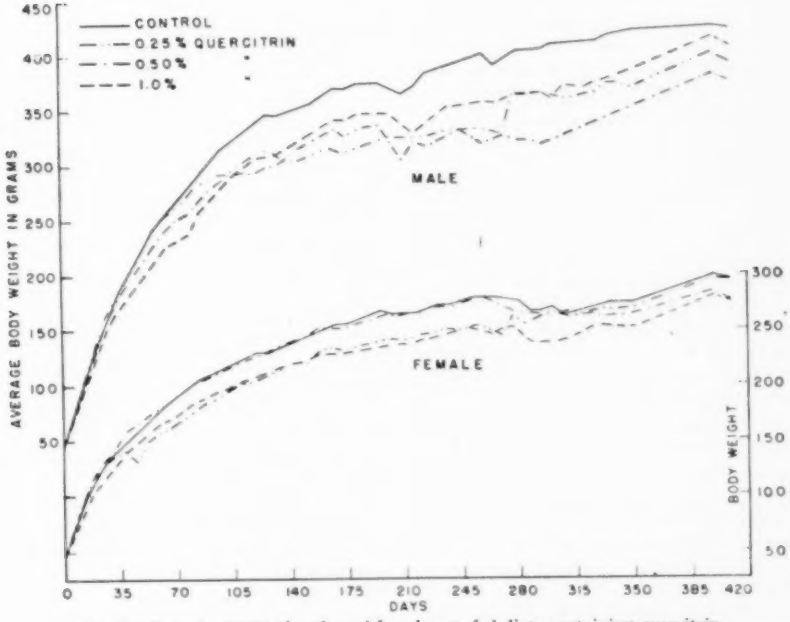


Fig. 2.—Growth curves of male and female rats fed diets containing quercitrin.

periment, it is reasonable to assume that quercetin and quercitrin are nontoxic. These toxicity data, together with the fact that the physiological effects of quercetin and quercitrin in experimental animals are the same as those of rutin, suggest the possibility that these compounds may be used therapeutically in place of rutin.

SUMMARY AND CONCLUSIONS

1. Rabbits receiving solubilized quercetin or quercitrin intravenously in single doses of 100 and 150 mg./Kg., respectively, showed no evidence of toxicity.

2. Following intravenous administration in rabbits, at least part of both quercetin and quercitrin is eliminated rapidly via the kidneys as judged by the gross criteria of staining paper yellow and the intensification of the color of the urine with sodium hydroxide.

3. Albino rats on diet containing 1 per cent quercetin or quercitrin or less for 410 days failed to produce evidence of abnormalities or injury as judged by growth, food consumption, red and white blood counts, hemoglobin estimation, organ weights, and histopathological examination of the tissues.

4. On the basis of experimental evidence presented, with rabbits and rats, it is concluded that quercetin and quercitrin have a low order of toxicity and it is doubtful that they exert any specific toxic effects.

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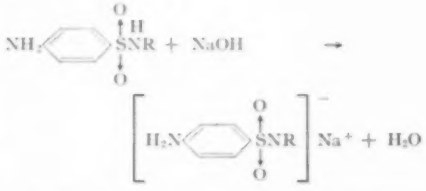
Spray-Drying of Insoluble Weakly Acidic Pharmaceuticals through Their Ammonium Salts*,†

By T. HIGUCHI, M. GUPTA,‡ and L. W. BUSSE

The theoretical feasibility of spray-drying weakly acidic pharmaceuticals after solubilization through their ammonium salts is shown. The practicability of this procedure is supported by its application to a number of sulfonamides and a barbiturate. A number of compounds do not follow the theoretical treatment because of their tendency to form complexes.

THE USEFULNESS of the spray-drying process in the production of pharmaceutical powders was pointed out in a previous report (1). The method described, however, had only limited application to the sulfonamide drugs because of the limited solubility of these compounds in water. The results of a recent investigation on possible methods of overcoming this obstacle are presented in this paper.

The solubilities of weakly acidic substances such as the sulfonamides can be greatly enhanced by the use of high *p*H aqueous solutions. Some of the mathematical relations involved have been recently reviewed by Higuchi (2). Permanent bases such as sodium or potassium hydroxide cannot be used, however, since spray-drying of solutions solubilized by these bases would yield the respective salts of the sulfa drugs, e. g.,



As shown in the following analysis, however, if a volatile base, e. g., ammonia, is used, it seemed possible that both the increased solubility and recovery of the product in the nonsalt form can be realized. One can expect the equilib-

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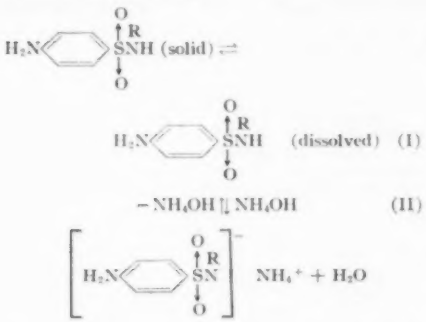
rium of a system containing ammonium hydroxide and a sulfonamide to be governed by equilibrium reaction I.



The equilibrium constant of reaction I is, of course, identical with the solubility of the original drug K_s. The equilibrium constant for reaction II is the hydrolysis constant shown in Eq. 1.

$$K_h = \frac{\left[\text{sulfonamide anion}\right][NH_4OH]}{\left[\text{sulfonamide anion}\right][NH_4^+]} = \frac{K_w}{K_a K_b} \quad (1)$$

By using Eq. 1 it is possible to approximate the amount of sulfa drug dissolved by any given initial amount of ammonium hydroxide. Thus for all practical purposes:

$$\left[\text{sulfonamide anion}\right] = [NH_4^+]$$

and since

$$\left[\text{sulfonamide}\right] = K_s,$$

$$\left[\text{sulfonamide anion}\right]^2 = \frac{K_a K_b K_s}{K_w}[NH_4OH]$$

and

$$\left[\text{sulfonamide anion}\right] = \sqrt{\frac{K_a K_b K_s}{K_w}[NH_4OH]} \quad (2)$$

Since K_s is fairly small for nearly all of the sulfa drugs, the last expression above gives essentially the relationship between the equilibrium concentration of ammonium hydroxide and the amount of the drug in solution. Furthermore, since:

$$[NH_4OH]_{\text{Initial}} = [NH_4OH] + \left[\text{NH}_2\text{-C}_6\text{H}_4\text{-SO}_2\text{NR}^-\right]$$

we can write (*next column*):

$$\left[\text{NH}_2\text{-C}_6\text{H}_4\text{-SO}_2\text{NR}^-\right] = \sqrt{\frac{K_a K_b K_s}{K_w}\left([NH_4OH]_{\text{Initial}}\left[-\text{NH}_2\text{-C}_6\text{H}_4\text{-SO}_2\text{NR}^-\right]\right)}$$

or

$$[NH_4OH]_{\text{Initial}} = \frac{K_w}{K_a K_b K_s}\left[\text{NH}_2\text{-C}_6\text{H}_4\text{-SO}_2\text{NR}^-\right]^2 + \left[\text{NH}_2\text{-C}_6\text{H}_4\text{-SO}_2\text{NR}^-\right] \quad (3)$$

In Fig. 1 the above expression is plotted to show the expected relationship between the initial ammonium hydroxide concentration based on the final value necessary to solubilize a given weight of sulfathiazole per 100 ml. of solution. It is evident that relatively highly concentrated solutions can be obtained by this method. The important question at this point is: would sulfonamides solubilized in this manner revert back to the original acidic form on spray-drying?

Although the data available are insufficient to define the system exactly, the above question can be answered in the affirmative on the basis of the following analysis:

It can be seen that in a system composed of the solid acidic form of the drug, the solid ammonium salt of the drug, and an aqueous phase in equilibrium with the two solid phases, the composition of the aqueous phase is an invariant at any given temperature. If an attempt was made to increase the ammonium hydroxide concentration by addition of the base to the system, the initial concentration would be restored by conversion of some of the acidic solid form to the solid ammonium salt form. It is evident that the converse would also be true. This ammonium hydroxide concentration can be calculated from Eq. 1 by making the substitutions shown in Eq. 4.

$$\left[\text{sulfonamide}\right] = K_s = \text{solubility of the drug,}$$

$$\left[\text{NH}_2\text{-C}_6\text{H}_4\text{-SO}_2\text{NR}^-\right] = K'_s = \text{solubility of the ammonium salt of the drug,}$$

and

$$[NH_4^+] = \left[\text{NH}_2\text{-C}_6\text{H}_4\text{-SO}_2\text{NR}^-\right] = K'_s$$

or

$$[NH_4OH] = \frac{K_w (K'_s)^2}{K_a K_b K_s} \quad (4)$$

Although the last expression given in Eq. 4 is far from being exact because of use of concentrations instead of activities, it can be expected to be at least approximately correct.

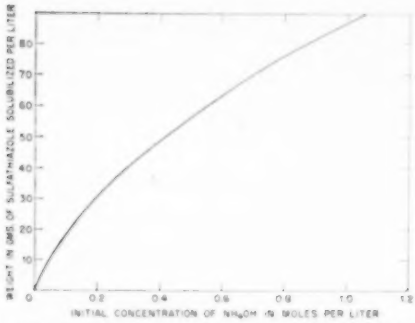


Fig. 1.—A curve showing the amount of sulfathiazole which can be solubilized by a given initial concentration of ammonium hydroxide at 25°.

It is evident that if, at this concentration of ammonia, the rate of escape of ammonia as a vapor is such that the combined concentrations of free ammonium hydroxide and ammonium ion decrease despite concomitant loss of water through evaporation, any undissolved ammonium salt of the sulfonamide will go into solution and the sulfonamide itself will be precipitated out. This would be the case if:

$$\frac{[NH_4OH]}{[NH_4^+]} \times R \gg 1 \qquad (5)$$

where R is the relative volatility ratio of ammonia and water. According to data obtained from International Critical Tables, it varies from roughly 15 at 100° to 30 at 30°. Or, substituting the value from ammonium hydroxide from Eq. 4 into the above, we get:

$$\frac{RK_wK'_s}{K_aK_bK_s} \text{ must be } \gg 1 \qquad (6)$$

to obtain only the free acidic form on spray-drying of the ammoniacal solution.

For sulfathiazole we can determine the numerical value of the above expression by substituting

$K_w = 1 \times 10^{-14}$ (ionization constant of water at 25°)
$K_a = 5 \times 10^{-8}$ (ionization constant of sulfathiazole at 25°)
$K_b = 2 \times 10^{-5}$ (ionization constant of ammonium hydroxide at 25°)
$K_s = 2 \times 10^{-3}$ (molar solubility of sulfathiazole at 25°)
$K'_s \cong 1$ (molar solubility of NH_4 sulfathiazole at 25°)
$R \cong 20$

or

$$\frac{RK_wK'_s}{K_aK_bK_s} \cong 1 \times 10^2 \gg 1$$

Actually the value of 100 is probably low, as K_w changes from 10^{-14} to nearly 10^{-12} at 100°. If we assume equilibrium conditions, we can reasonably expect, therefore, that sulfathiazole will revert completely to its original form on spray-drying from ammoniacal solutions.

EXPERIMENTAL

The practical feasibility of the use of ammonia as a solubilizing agent for spray-drying of weakly acidic drugs was established by applying the method to sulfathiazole, sulfadiazine, and a nonsulfa type drug, barbital.

Sulfathiazole.—150 Gm. of U. S. P. sulfathiazole was dissolved in 3.5 L. of water containing 95 ml. of concentrated ammonium hydroxide. This solution was filtered and spray-dried on a Laboratory Model Bowen Spray-Drier at the rate of 30 ml./min. The gas inlet temperature was maintained at 205° and the outlet temperature at 52°.

The spray-dried product was shown by its melting point (197–199°) to be sulfathiazole. The absence of any ammonium salt in the product was established by back titration of a volume of standard acid solution used to catch the distillate from an alkaline solution of a known weight of the drug.

Sulfadiazine.—The procedure outlined above also yielded spray-dried sulfadiazine free from ammonium ions.

Barbital.—The method was also applied to barbital to illustrate its possible applications to drugs other than the sulfas.

Ninety grams of U. S. P. barbital was dissolved in 1 L. of water containing 65 ml. of concentrated ammonium hydroxide. The solution was filtered and spray-dried as before.

The product by its melting point and analysis proved to be free of ammonium barbital. The ammonium ion content was determined by titration in glacial acetic acid with perchloric acid and was found to be less than 0.01%.

Some Acids Which Yield Ammonium Salts.—As may be expected from Eq. 6, stronger and more water-soluble acids cannot be solubilized and spray-dried without obtaining their ammonium salts. Barbituric acid, for example, was found to yield spray-dried product containing 97–98% ammonium barbiturate. Barbituric acid is roughly 1,000 times stronger acid than barbital, making $RK_wK'_s/K_aK_bK_s$ less than one.

Benzoic acid was also expected to yield its ammonium salt because of its great acidic strength. However, since benzoic acid forms a relatively insoluble complex with ammonium benzoate, spray-drying produced a powder which was found to be approximately one-half salt. Elucidation of this phenomenon will be reported elsewhere.

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(2) Higuchi, T., "Pharmaceutical Compounding and Dispensing," J. B. Lippincott Co., Philadelphia, 1949, p. 179.

Determination of Certain Properties of Ointment Bases by Use of Chick Embryos*

By MATTHEW C. HUNTER† and F. JANE SMITH

A new method is presented for the evaluation of certain properties of ointment vehicles. This method can be used to measure the release of an active agent incorporated in various vehicles and permeability of the agent, as well as the base, through ductless membranes.

THE EFFICACY of therapeutic agents incorporated in ointment bases is a subject which has received considerable experimental and empirical analysis in the past few years. Many ingenious methods have been proposed in attempts to evaluate the *in vivo* value of the various bases by both *in vitro* and *in vivo* methods, but the problem is still far from its ultimate solution.

Too frequently conclusions have been drawn concerning the value of certain ointments because specific ones demonstrated superiority in some single aspect. Actually, the usefulness of any topically applied preparation is dependent upon numerous variable factors. Frequently bases selected because they were shown to be best for one therapeutic agent were found subsequently to be ineffective for a different agent.

HISTORICAL BACKGROUND

Before 1931 few comparative studies were made of the specific properties of ointment bases conducive to clinical efficacy. Ruehle and Brewer (1) then proposed the agar plate method for evaluation of bacteriostatic ointments. This method has been modified by numerous investigators (2–6) and has stimulated considerable thought and constructive criticism.

The determination of the degree of liberation of sulfonamides in an aqueous phase (7–11) and penicillin in an aqueous or serum phase (12) also contributed considerably to the evaluation of various ointment bases. Measurements of the dialysis of active ingredients through cellophane (12) or other semipermeable membranes (13, 14) have supplied valuable information on the rate of release.

Although the methods mentioned above contributed suggestions as to the penetrability and release of active ingredients from ointment bases, no direct correlation could be made between the results of such investigations and the ultimate therapeutic efficacy. Blood levels were used to measure the penetration of topically applied sulfonamides by Zondek, *et al.* (15), and Magner and O'Sullivan (10). In 1943 Rothman (16) presented an excellent summary of percutaneous absorption and the features affecting it. Histochemical methods for the demonstration of sulfonamide and other drugs in the skin revealed the routes of entry and allowed for a qualitative estimate of degree of penetration (17, 18). In 1943 Johnston and Lee (19) suggested radioactive tracer compounds to follow skin absorption, and recent investigations (20, 21) indicate that this method is quite applicable. Woodard, *et al.* (22) evaluated the penetration of sulfonamides into intact rabbit skin by direct chemical analysis of skin biopsies, as well as by blood and urine analysis. An *in vitro* method of measuring skin penetrability has been proposed by Schwartz, *et al.* (23), and has been modified by Eisner (24).

The *in vitro* methods which have been devised can contribute to our knowledge of a number of the specific properties of ointment bases but cannot be directly correlated with *in vivo* efficacy. The *in vivo* methods which are now available are applicable in many cases but are somewhat limited. Large numbers of experimental animals are necessary to make comparisons of ointments which can be evaluated statistically. Many ingredients cannot be assayed accurately, if at all, when diluted by the body fluids. Since each ointment base may bind various constituents to a greater or lesser degree, results obtained with a single base for two different active agents may vary considerably, and a large number of experimental animals would be required to make a systematic and statistically reliable investigation.

In an attempt to make a comparative evaluation of a number of ointment bases which might serve effectively as vehicles for an antifungal agent, we were faced with the problem of determining which base would allow the most rapid release of the therapeutic agent and the greatest skin penetration directly, that is, excluding penetration through glandular ducts.

All of the methods in present use measure the penetrability of an agent through the skin primarily by means of the hair follicles and glands and not directly through the superficial stratified epithelial layers. Since none of the methods seemed feasible for our purpose, we attempted to devise one in which we would utilize a ductless membrane.

CHICK EMBRYO METHOD

A method utilizing the chick embryo seemed most applicable for this purpose. The membranes through which we attempted to measure release and penetration of drugs are the shell membrane, a thin fibrous nonliving coagulum, and the chorio-allantoic membrane.

The shell membrane lines, and is closely adherent to, the shell. The membrane is tough in consistency and has fibers running roughly in the long axis of the

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egg, but somewhat obliquely. At the broad end of the egg is the air sac, a well-developed space entirely enclosed by shell membrane, the layers of which divide to form the space. The air sac is small in sterile eggs, but becomes considerably larger in fertile eggs.

The chorio-allantoic membrane is composed of three layers, each of which represents one of the primary germinal layers. The outer or ectodermal layer consists of a single layer of chorionic epithelium. Directly beneath it is the mesoderm, representing a fusion of chorionic and allantoic mesoderm. This mesodermal layer, through which small blood vessels traverse, consists of a single layer of cells. The entoderm is made up of the inner lining of the allantois and consists of a single layer of cells (25).

When sections are made of the chorio-allantoic membrane in normal contact with the shell membrane at about the twelfth day of incubation, the structure observed indicates clearly the predominantly respiratory function of the membrane. The main capillary network lies immediately against the ectodermal cells, and it is difficult to determine whether the capillaries are actually in contact with the shell membrane or separated from it by very flattened ectodermal cells.

Immediately under this chorio-allantoic membrane is the allantoic fluid. The amount of fluid varies from about 1 cc. to about 6 or 7 cc., depending primarily upon the age of the chick embryo (26).

Such a tissue system is thin enough to allow rapid assays of the properties of various vehicles. Small quantities can be measured. Chick embryos are inexpensive enough to permit use of numbers statistically valid and the techniques to be described are simple enough to allow complete assays to be carried out in a short period of time by inexperienced technicians.

Chick Embryos.—All of the embryonated eggs used were from white leghorn hens and averaged 2 oz. or more per egg. All embryonated eggs were candled and the shells cut on the day of delivery and reincubated until the next day. They were then recandled and any eggs which showed any undesirable effect from cutting were discarded. The suitable eggs were inoculated either directly into the allantoic cavity or between the two layers of shell membrane in the natural air sac. Inoculated eggs were reincubated and observed periodically for death. When necessary, the allantoic sac contents were withdrawn immediately after death was observed and were assayed microbiologically.

Methods of Inoculation.—When assaying the penetrating power of an ointment through the chick membrane, the following method was used. When the eggs were nine days old a small hole (2–3 mm. in diameter) was drilled directly above the natural air sac through the shell but not through the underlying shell membrane. The following day a 1.0-cc. tuberculin syringe was filled with the ointment to be assayed; a No. 16 or 18 inoculating needle placed on the syringe, and the air in the needle displaced. The shell membrane adherent to the shell was punctured and a measured amount of ointment ejected onto the shell membrane adherent to the chorio-allantois. After withdrawal of the needle, the hole through the shell was sealed with melted paraffin or scoeth tape, and the eggs were reincubated.

When assaying the chick embryo toxicity of complete ointments or individual constituents of ointments, the following technique was employed. While candling the eggs, areas were marked on the shell immediately above the chorio-allantois in well-developed areas. Care was taken to avoid cutting the shell immediately above any of the discernible blood vessels so as to avoid hemorrhage. In the marked space a small hole was then cut through the shell but not through any of the underlying membranes. The holes were then sealed with melted paraffin and the eggs reincubated. The next day all eggs were recandled and all those which showed any hemorrhage were discarded. Preparation of syringes and inoculation procedures were identical to those described above.

Ointments.—The five ointments shown in Table I, with and without 2,000 u./Gm. of antibiotic XG[1] were tested for release of antibiotic XG and for tissue penetrability.

TABLE I.—OINTMENTS INVESTIGATED

Ointment	Composition	%
Oil in water	Stearyl alcohol	7.0
	Cetyl alcohol	3.0
	Sodium lauryl sulfate	1.0
	Mineral oil (light)	10.0
	Polyethylene glycol 4000	20.0
	Sodium phosphate U. S. P.	0.1
	Water	58.9
Waterless gel	Propylene glycol dipropionate	87.0
	Ethocel, 209 c. p. s.	10.0
	Tween 20	3.0
Carbowax base	Polyethylene glycol 4000	45.0
	Propylene glycol	55.0
Grease base 1	G-1425, Atlas[a]	25.0
	White petrolatum	75.0
Grease base 2	Sodium lauryl sulfate	2.0
	Mineral oil (light)	39.5
	Petrolatum	30.0
	Cetyl alcohol	11.0
	Stearyl alcohol	17.0
	Titanium oxide	0.5

[a] Polyoxyethylene sorbitol lanolin derivative.

EXPERIMENTAL

Determination of Optimum Age of Chick Embryo.—In order to learn the ideal stage of development of the chick embryo for use in determining efficacy of various ointment bases, we tested embryos from four to nineteen days old. The inoculum consisted of approximately 0.25 Gm. of Carbowax base or of Carbowax base plus 500 units of antibiotic XG. The inoculum was deposited upon the surface of the shell membrane by ejection from a tuberculin syringe. The chicks were examined every two hours and the time of death recorded. Five chick embryos at each of nine ages were used for each of the two ointments. Since Carbowax is quite hygroscopic, fluid was withdrawn from the allantoic cavity by the Carbowax until an equilibrium was reached and

[1] An amphoteric polypeptide supplied by Charles Pfizer & Co. under the trademark "Fungistatin."

then there was an exchange of fluids between the aqueous solution of Carbowax and the residual allantoic fluids. Death of the embryo preceded the depletion of Carbowax solution from the surface of the membranes, allowing assay of the surface fluid as well as the allantoic fluid for antibiotic XG. The average death time for each group of chicks is recorded in Fig. 1.

In all of the chicks between the ages of four and thirteen days, which were inoculated with the ointment containing antibiotic XG, there were between 250 and 300 units of antibiotic in the surface fluids. Evidently from 200 to 250 units of antibiotic penetrated the membrane to produce death of the chick embryo. The older the chick embryos the smaller the quantities of antibiotic found in the surface fluid and the larger the quantity of antibiotic in the allantoic fluid at the time of death.

It is very likely that in the above experiment both the antibiotic and the Carbowax base penetrated the extra-embryonic and shell membranes. In those cases where the bases containing the antibiotic produced death before those not containing the antibiotic, we can logically assume that enough of the antibiotic penetrated to produce death before enough of the Carbowax penetrated to produce death—since the antibiotic is much more toxic to the chick embryo than the Carbowax.

The younger the chick embryos the wider the variation in death times caused by Carbowax only and Carbowax plus antibiotic; however, the younger embryos could not be analyzed for viability as readily as the older ones. Embryos which were ten days old were easily observed for viability and still possessed membranes of only slightly greater permeability than those of the youngest chicks, so they were considered of optimum age for our other experiments.

Since the extra-embryonic membranes of the older chick embryos are provided with a much richer supply of blood vessels, the question arose concerning the route through which the toxic agent reached the chick embryo to cause death. Was the agent absorbed directly into the blood vessels and then carried to the chick embryo or did the agent diffuse through the membranes into the allantoic fluid and then into the chick embryo?

If the agent did gain access to the embryo via the chorioallantoic blood vessels, a large amount of the antibiotic should localize in the embryonic tissue and only gradually be released into the allantoic fluids. A number of chicks were inoculated with antibiotic XG in saline on the surface of the shell membrane. Immediately after the death of each chick embryo, samples were taken of the allantoic fluid and of the yolk sac material and then the embryo proper was washed and suspended in a small quantity of sterile saline. After all chick embryos had died the samples were pooled and the embryos homogenized in a Waring blendor. Each of these materials was assayed for antibiotic XG. No antibiotic could be detected in the embryo or in the yolk sac but it was present in high concentration in the allantoic fluid. Since a toxin present only in the allantoic fluid would not kill the chick embryo, some of the material must have been present in the embryo or in the extra-embryonic circulation,

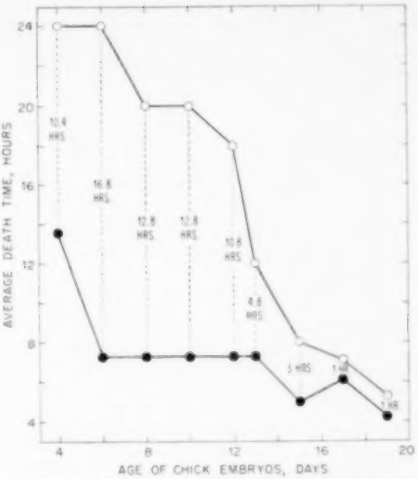

Fig. 1.—Variation in permeability of extra-embryonic membranes of chick embryos at various stages of development.
○——○ Carbowax ointment base without antibiotic XG.
●——● Carbowax base containing 2,000 units antibiotic XG per Gm.

but was present in such small amounts that it could not be detected.

In another experiment in which saline solutions of antibiotic XG were injected either directly into the allantoic cavity or upon the external surface of the extra-embryonic membrane, it was shown that: (*a*) with equal quantities of antibiotic XG, chick embryos consistently died sooner when the material was injected directly into the allantoic cavity, and (*b*) the amount of antibiotic XG which could be recovered from the allantoic fluid immediately after death was always smaller in those embryonated eggs in which the total amount was originally deposited directly in the allantoic fluid.

Thus it has been demonstrated that extremely small quantities of antibiotic XG were lethal to the chick embryo; that death was produced more rapidly by inoculation directly into the allantoic cavity than by inoculation upon the external surface of the membranes; that large quantities of antibiotic XG were present in the allantoic fluid at time of death; that the amounts of antibiotic XG present in the chick embryo and yolk material at time of death were so small that they cannot be detected by microbiological assay; and that antibiotic XG was rapidly absorbed by the embryo from the allantoic fluid. In view of these facts, it appeared reasonably certain that death was caused primarily by absorption of the toxic agent from the allantoic fluid rather than by the chorio-allantoic circulation. More rapid death in older embryos suggested that the increased number of blood vessels disrupted the uniformity of the membranes and thereby enhanced permeability.

Effect of Ointments Injected into Allantoic Cavity.—Preliminary experiments indicated that the ointments chosen for testing were toxic to the chick

TABLE II.—EMBRYOCIDAL ACTIVITY OF OINTMENTS INJECTED INTO ALLANTOIC CAVITY

Ointment	Amount Injected, Ml.	No. Chicks Used	Chicks Remaining Alive after: 2 Hr.	4 Hr.	6 Hr.	24 Hr.	48 Hr.	6 Days
Oil-in-water	0.1	10	10	10	10	10	10	10
Waterless gel	0.1	10	6	2	0	0	0	0
Carbowax	0.1	10	10	10	10	9	6	6
Grease base 1	0.1	10	10	10	10	10	10	10
Grease base 2	0.1	10	10	10	10	10	10	10

embryo. Each base was therefore checked for the acute toxicity by injecting the ointment directly into the allantoic cavity.

The ointments were warmed to approximately 40° in a water bath and drawn or pushed into 1.0-cc. tuberculin syringes. Inoculating needles (No. 20) were used to inject 0.1 Gm. of base into the allantoic cavity of the chick. The holes in the shells were sealed with sterile paraffin and the chicks were reincubated. Chick embryos were observed at two-hour intervals for sixteen hours and daily thereafter for seven days. The results are recorded in Table II.

Since the waterless gel ointment was quite toxic to the chick embryo, twofold dilutions of pure propylene glycol dipropionate were prepared and injected into another series of eggs. Each embryonated egg received 0.1 cc. of one of the dilutions. The results of the experiment are recorded in Table III.

It is evident from the results recorded in Table III that all chick embryos were killed by between 0.1 cc. of the 1:2 dilution and 0.1 cc. of the 1:4 dilution of propylene glycol dipropionate, when injected directly into the allantoic cavity. The LD_{50} of propylene glycol dipropionate calculated by the method of Reed and Muench (27) was approximately 0.02 cc.

It became apparent, in the light of these results, that we would have to exercise caution in interpreting results of subsequent experiments with the waterless gel base and Carbowax base. A control (without antibiotic XG) was considered necessary for each ointment in order to evaluate accurately whether death of the chick embryo was being caused by penetration of the ointment base only or penetration of both the ointment base and the antibiotic with subsequent release of the antibiotic.

Effect of Ointments on the Surface Membranes of the Chick Embryos.—In order to determine whether death could result from harm inflicted on the tissues of the extra-embryonic membranes by the ointments without antibiotic XG, a series of embryonated eggs was inoculated with 0.5 Gm. of each ointment base on the surface of the shell membrane. All eggs were immediately reincubated and were observed every two hours for the first sixteen hours and daily thereafter for four days.

The propylene glycol dipropionate base rapidly penetrated the extra-embryonic membranes and was lethal to the chick embryos. No grossly discernible hemorrhage was observed in the vessels of the chorio-allantois and no ointment remained on the external surface of the membranes.

The Carbowax base caused death to occur in all chick embryos in an average time of thirteen hours. Gross hemorrhage was evident in the chorio-allantoic blood vessels in the area where the ointment had been placed, but vessels which had not been in direct contact with the ointment appeared normal.

Penetration of grease base 1 and grease base 2 was evidently insignificant, since the average death times were well over one hundred hours in both cases. Actually most chicks in each of these groups hatched normally about twelve days after inoculation. This indicated that interference with normal respiration of the chick embryo by the block of a portion of the respiring membrane played no significant part in causing the death of the chick embryo. In no case was any injury observed which could be attributed to either of the grease bases.

The oil-in-water ointment also showed slight if any penetration, as was evidenced by almost total recovery of the ointment from the external surface of the membrane. This base, as well as the two petrolatum-containing bases, was solid on the surface of the membranes as originally deposited there and there was no indication of injury to the underlying membranes.

Relationship between Concentration of Toxic Agent and Survival Time.—In order to gain a better conception of the relationship between the concentration of toxic agent present in the allantoic cavity and the survival time, chick embryos were injected with various concentrations of antibiotic XG by various routes, and the time of death observed and the amount of antibiotic in the allantoic fluid recorded.

Fifty chick embryos were divided into five groups. The first group received 0.4 cc. of saline containing 200 units of antibiotic on the external surface of the shell membrane. The second group received an identical inoculum by injection directly into the allantoic cavity. The third group received 0.4 cc. of saline containing 1,000 units of antibiotic on the

TABLE III.—CHICK EMBRYO TOXICITY OF PROPYLENE GLYCOL DIPROPIONATE (PGDP)

No. Chicks Used	Dilu. of PGDP	Chicks Remaining Alive after: 2 Hr.	4 Hr.	6 Hr.	8 Hr.	10 Hr.	12 Hr.	24 Hr.	48 Hr.	148 Hr.
10	Pure	6	2	2	2	2	2	0	0	0
10	1:2	8	8	6	6	6	6	0	0	0
10	1:4	8	8	8	6	6	6	5	5	4
10	1:8	10	10	10	10	10	10	10	10	10
10	Control	10	10	10	10	10	10	10	10	10

external surface of the shell membrane, and the fourth group received an identical inoculum by injection directly into the allantoic cavity. The fifth group received 0.4 cc. of saline only by injection directly into the allantoic cavity and served as a control.

Immediately after death was observed in each egg, a sample of the allantoic fluid was withdrawn and assayed for antibiotic XG activity. Averages of death time and concentration of antibiotic XG in the allantoic fluid are recorded in Table IV.

Since the amounts of allantoic fluid in the chick embryos were not measured, no direct correlation can be made between the amounts of antibiotic XG found in the allantoic fluid and the amounts absorbed by the embryo before death occurred. Since, however, in two cases an identical quantity of antibiotic was either injected into the allantoic cavity or placed on the surface of the shell membrane, and in both cases greater amounts of antibiotic were found in the allantoic fluids of the groups which were inoculated by placing the antibiotic on the surface membranes, it can be logically assumed that chick embryos absorbed the antibiotic from the allantoic fluid and that death ensued. It can also be assumed that since death occurred significantly sooner when injections were made directly into the allantoic cavity, that death was not due to the harm imposed upon the blood vessels present in the chorio-allantoic membranes by the antibiotic. Therefore, the probable course of the antibiotic when placed on the surface of the shell membrane was penetration through the shell and chorio-allantoic membranes, distribution in the allantoic fluid, and absorption from the allantoic fluid by the chick embryo until a sufficient quantity had been absorbed to produce death.

It may be concluded from these experiments that death does not occur immediately after penetration of the antibiotic through the extra-embryonic membranes, but only after enough time has elapsed to allow absorption by the embryo. The time required for absorption of a lethal dose is dependent to some extent upon the concentration of the antibiotic in the allantoic fluid.

Comparison of Ointment Bases.—After accumulating this basic information on the effects of the antibiotic and various bases on the chick embryo by various routes of inoculation, it was possible to evaluate more critically the results of combinations.

Fifty chick embryos were divided into five groups. Each group was inoculated with 0.5 Gm. of one of the five ointments, each of which contained 2,000 units of antibiotic XG per Gm. All inoculations were made by ejecting the ointments directly onto the external surfaces of the shell membranes. Observations were made every two hours for the first twenty-four hours and once a day thereafter for eight days.

Table V shows a comparison between the times required to produce death of the chick embryo by the ointment bases alone and the ointment bases containing antibiotic XG.

Death of the embryos tested with the waterless gel base with and without antibiotic XG occurred in two hours. The propylene glycol dipropionate present in the ointment was so toxic that no differential in survival time could be obtained between the base alone and the base plus the antibiotic XG. However, since antibiotic XG was recovered from the allantoic fluid immediately after death of the embryos, it was definitely carried along by the rapidly penetrating waterless gel base.

TABLE IV.—AVERAGE DEATH TIME AND UNITS OF ANTIBIOTIC XG PER ML. OF ALLANTOIC FLUID IN EMBRYOS INOCULATED WITH ANTIBIOTIC XG IN SALINE

Route of Inoculation	Units of XG in Inoculum	Death Time	Units XG/Ml. of Allantoic Fluid
Shell membrane	200	187 min.	38
Allantoic cavity	200	70 min.	25
Shell membrane	1,000	142 min.	252
Allantoic cavity	1,000	43 min.	235
Allantoic cavity	0	No deaths in 8 days	...

Carbowax base only and Carbowax base plus antibiotic XG produced deaths of the embryos, as would be expected from the results of previous experiments (Fig. 1). In this case a very significant differential in survival time was obtained which proved that antibiotic XG was carried along with the ointment through the extra-embryonic membranes. In addition, antibiotic XG was shown to be present, by microbiological assay, in allantoic fluids of chick embryos which died significantly sooner than those exposed to Carbowax only.

The oil-in-water base and the oil-in-water base plus antibiotic XG gave results quite different from those shown by most *in vitro* methods. This base was expected to penetrate fairly rapidly, but actually penetrated more like the petrolatum-containing bases than like the Carbowax base.

Grease base 1 penetrated, if at all, at such a slow rate that it could not be measured by this method over a period of one hundred and ninety-two hours. The allantoic fluid of these chick embryos did not contain a measurable quantity of antibiotic XG.

Grease base 2 evidently penetrated the membranes, but did so at a very slow rate, as evidenced by an average survival time of almost five days.

DISCUSSION

In interpreting results obtained through the use of the chick embryo technique, certain limiting factors should be taken into consideration. Foremost of these is the lack of keratin in the nonliving shell membrane and the chorio-al-

TABLE V.—COMPARISON OF SURVIVAL TIME OF EMBRYOS INOCULATED WITH BASE ONLY AND WITH BASE CONTAINING ANTIBIOTIC XG

Ointment	Death Time from Base Only, Hr.	Death Time from Base and XG, Hr.
Oil-in-water	192	84.5
Waterless gel	2	2.0
Carbowax	13	4.0
Grease base 1	192	192.0
Grease base 2	192	117.5

lantoic membrane. Also of possible significance is the embryonic nature of the tissues making up the chorio-allantois. The three primary germ layers found in the chorio-allantois are in contrast to the single ectodermal layer which gives rise to the epidermis of the adult body. A further consideration is the remote phylogenetic relationship between the birds and mammals. The degree of influence that these features of the test may have on the validity of the results is difficult to ascertain, but they probably cause some variation in the mechanism of transmission of an agent through this tissue system as compared with the adult human epidermis.

The direct transfer of results from the test tube or the experimental animal to the clinic always carries the possibility of error. As a general rule, however, the more closely the experimental methods simulate the prevailing clinical conditions the more applicable are the results. The ideal technique for routine testing is one which simulates most closely the final application and is still both adaptable and practical.

Most of the techniques previously proposed for the evaluation of ointment bases have been both adaptable and practical, but were generally of such a nature that they measured only one or two of the many properties which may contribute to the efficacy of the preparation. Methods of analysis are known which are more or less effective for measuring the ability of an ointment to traverse, through the hair follicles or glandular ducts, into the connective tissue underlying the keratinized epithelial layer, but no practical methods have yet been described which measure the ability of an agent to permeate directly through a homogeneous sheet of tissue as is available in the embryonated egg.

The salient features of the chick embryo technique which provide definite advantages over previously proposed methods for the evaluation of vehicles for antifungal agents are: (*a*) use of a living membrane devoid of glandular ducts or hair follicles, (*b*) use of living tissue covered by a relatively dry, nonliving membrane (shell membrane), (*c*) use of a membrane thin enough to allow for the rapid analysis of various types of active ingredients from various types of vehicles, (*d*) use of a biological system in which very small samples can be assayed *in vivo* without concern for problems of excretion, (*e*) use of a technique simple enough to be mastered by inexperienced personnel with a minimum of technical supervision, and (*f*) use of readily available and inexpensive experimental subjects, thereby permitting the use of statistically valid numbers.

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A Cylinder Dispenser for Use in Agar Diffusion Assays*

By E. H. CHRISTIANSEN and W. A. ZYGMUNT

A cylinder dispenser for use in agar diffusion assays for biological determination of activity of antibiotics and of vitamin B_{12} is described and its construction is illustrated. The apparatus was designed to overcome certain inherent difficulties encountered in the use of other available mechanical devices for dropping the required number of assay cylinders per plate.

USE OF THE AGAR diffusion assay procedure in microbiological estimation of antibiotic and vitamin B_{12} potency has been widespread in recent years. In contrast with acidimetric and turbidimetric assay procedures, the method herein described has the practical advantages of ease of manipulation, and greater number of assays per technician.

Since the basis of the assay method is diffusion, the placement of the assay cylinders on the agar surface is critical. That is, a uniform seal of the cylinder and agar surface is essential. In a small-scale operation, the cylinders can be placed individually in the desired position manually.

The advent of mechanical devices for dropping the required number, usually six, of assay cylinders per plate introduced an important time- and labor-saving element (1, 2). These devices have certain limitations, however. Loading, sterilizing, and interchanging of the magazines holding the assay cylinders is a cumbersome task. Secondly, two separate mechanical manipulations are required for each plate being "cup set."

APPARATUS DESIGN

An apparatus (Figs. 1 and 3) was constructed by one of us (E. H. C.) which minimizes the above difficulties. The stainless steel assay cylinders are sterilized in bulk and placed in a plastic hopper (part No. 2) with a 115-volt/0.43-ampere motor[1] providing the necessary agitating force through a pulley attached to part No. 4. Part No. 3 which is stationary serves as a guide for feeding the cylinders into six hollow brass tubes (part No. 18) approximately three inches in length and accommodating eight cylinders per tube at one given time. A series of stops and locks (detail section A and B, Fig. 2) hold the cylinders in place. Depression of the lever (part No. 5) causes the release of six cylinders simultaneously, while a Petri dish is held in a stationary position. The apparatus is constructed essentially of brass, steel, and plastic components. No mechanical difficulties have been encountered in routine

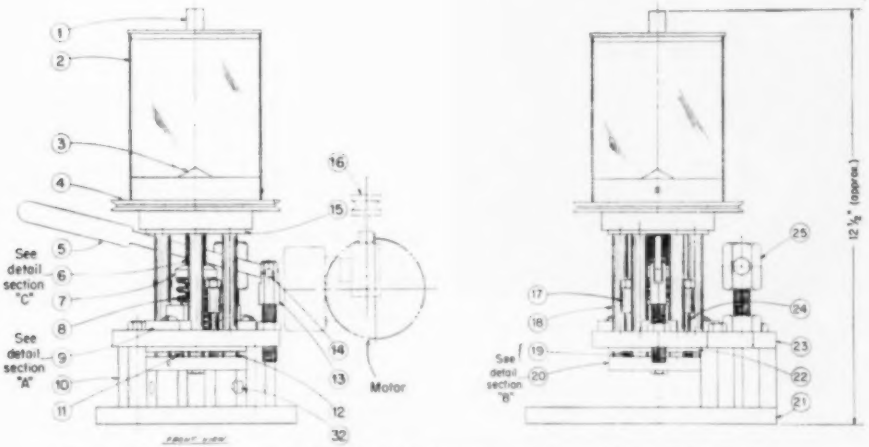

Fig. 1.—Sketch of front and right side of basic components comprising cylinder dispenser.

* Received October 12, 1951, from the Research Laboratories of Merck and Company, Rahway, N. J.

[1] The motor used was an 1/30-hp. gear head syn. model 4-620 American Instrument Co., 115 v., 0.43 amp., 500 r. p. m., ratio 10:1, torque 0.11.

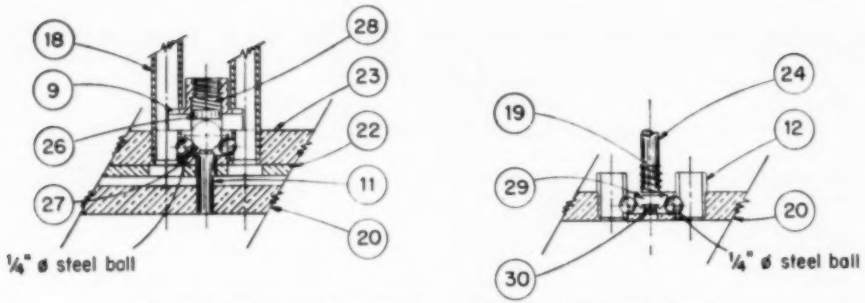

Fig. 2.—Detail sketch of section A and section B of cylinder dispenser.

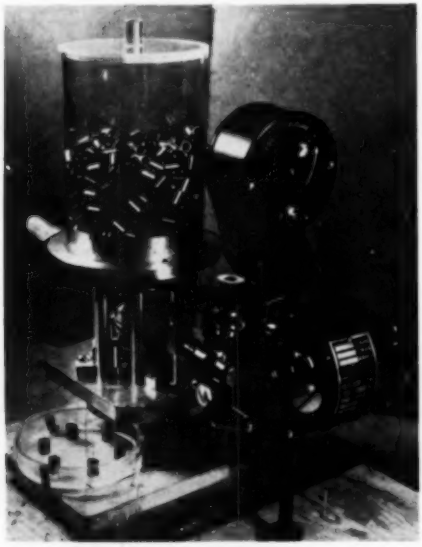

Fig. 3.—Photograph of cylinder dispenser in routine operation.

operation of the apparatus for a number of months. In essence, the basic principles involved are as follows:

Position of Rest

(A) When lever No. 5 is in a normal position (front view sketch), the cylinders in tubes No. 18 rest on plate No. 20 (detail section A) in a fixed position through the spring action depicted in detail section B.

Position of Operation

(A) Depression of lever No. 5 and the subsequent compression of spring No. 8 causes spindle No. 6 and plate No. 20 (detail section B, Fig. 2) to be lowered into the Petri dish in question.

(B) At the downward stroke of plate No. 20 and pin No. 11, pressure is applied on two $^1/_4$-inch diameter steel balls through the action of part No. 28 and causes the steel balls to hold the cylinders in a fixed position (detail section A, Fig. 2).

(C) As plate No. 20 is depressed the adjustable stops, No. 17, come to rest on plate No. 23.

(D) Plate No. 20 is further lowered approximately $^1/_{32}$ inch, as previously determined by adjustment of nut No. 7 on spindle No. 6, with the result that the pressure is relieved on the cylinders held in position as described under position of rest.

Position of Return

(A) Upon return of lever No. 5 to a position of rest, pressure is reapplied on the unit as depicted in detail section B through the action of spring tension between parts No. 19 and No. 22.

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Use of Bromsulphalein for Assay of Cobra Venom*

By GEORGE S. WARNER, JOHN H. BREWER, and H. A. BROWN DUNNING, Jr.

A colorimetric test is described for certain chemical constituents in cobra (Naja) venom, which are proportional to the toxic index in mice. The method consists of measuring the color intensity of an alkaline solution of the precipitate complex formed by an acid solution of Bromsulphalein with cobra venom.

THE USE of dyes for determination of free acid and basic groups in proteins has been reported (3–6). Colorimetric determination of the dye concentration bound in the precipitate of the protein-dye complex, which depends on the tendency of the polar groups of the protein to bind dyes of opposite charge in buffered solutions, was reported to agree with other protein titration methods (4). Disodium phenoltetrabromophthalein disulfonate (Bromsulphalein) was noted to precipitate casein completely from an acid solution, and the color intensity of an alkaline solution of the precipitate-complex was proportional to the amount of protein in the solution (2). Use of Bromsulphalein was extended by Greif (2) to the measurement of proteolytic activity and the quantitative determination of minute amounts of protein. Preliminary investigation in this laboratory indicated that a precipitate complex was formed by solutions of cobra venom[1] with buffered Bromsulphalein.

Present assay methods of the neurotoxic principle in cobra venom are animal toxicity tests. Specifically, our method is based on a lethal dose for a 20-Gm. albino mouse. Such methods have a possible disadvantage of measuring a fraction which may not be identical with the physiological activity of cobra venom. A simple colorimetric method which measures certain chemical constituents offers an additional assay method for biological standardization and study of cobra venom. Also, it provides for a reduction in time and animals required by animal assay. Colorimetric titration does not obviate a toxicity test to record a "safety factor" (1) which represents the magnitude of the dose as compared to the equivalent human dose.

This investigation reports the relationship between color intensity derived from an alkaline solution of the precipitate complex and the number of mouse units, as ascertained by mouse toxicity, per volume of cobra venom employed in the test.

EXPERIMENTAL

Materials.—Six lots of dried crude cobra (Naja) venom, designated A, B, C, D, E, and F, were selected, and a 500-ml. saline solution containing 250 mg. of crude venom was prepared from each lot. Each solution was rendered clinically acceptable by removal in large measure of the objectionable hemotoxin. Purified venom solutions were formulated with tricresol (0.3%) and were sterilized. Lot C-2 Special, which contained sufficient venom to assay 100 mouse units per ml. by mouse toxicity tests, was prepared in the manner stated above and was employed as the standard cobra venom solution.

The indicator solution was made by adding 1.5 ml. of 5% Bromsulphalein[2] to 98.5 ml. of citrate-phosphate buffer (McIlvain's) at *p*H 2.5.

Mouse Unit.—Number of mouse units in a cobra venom solution was determined by intraperitoneal injection of 20-Gm. albino mice. Mouse units were expressed in number per ml. A mouse unit was defined previously as the smallest amount of cobra venom required to cause the death of a 20-Gm. mouse in twenty-four hours.

Standard Bromsulphalein Curve.—Concentrations of Bromsulphalein covering the range 2–20 mg./L. were dissolved in distilled water, made alkaline with 2 ml. of 10% sodium hydroxide and diluted to volume with distilled water. The color intensity of each alkaline solution was recorded on a Fisher Electrophotometer using No. 525B (green) filter.

Bromosulphalein Precipitation Method.—The method employed was essentially that of Greif (2). The details of the method were: To a 15-ml. centrifuge tube, 1 ml. of a selected cobra venom solution was added. One milliliter of 0.85% sodium chloride solution and 2 ml. of indicator solution were added, and the entire volume mixed thoroughly and allowed to stand at room temperature for ten minutes. The resulting precipitate was centrifuged at approximately 2,500 r. p. m. for fifteen minutes. The supernatant liquid was discarded, and the tube was inverted and drained for several minutes. The inner wall of the tube was wiped dry with absorbent cotton on applicator sticks, and the precipitate was dissolved with 2 ml. of 10% sodium hydroxide solution. The alkaline solution was transferred quantitatively to a 25-ml. volumetric flask and made to volume with distilled water. Flasks were shaken thoroughly and the color intensity of each solution was determined on a Fisher Electrophotometer with

* Received October 31, 1951, from the Research Laboratories, Hynson, Westcott & Dunning, Inc., Baltimore, Md.

[1] Cobra (Naja) Venom Solution, Hynson, Westcott & Dunning, Inc.

[2] Hynson, Westcott & Dunning brand of 5% aqueous sulfobromophthalein sodium, U. S. P.

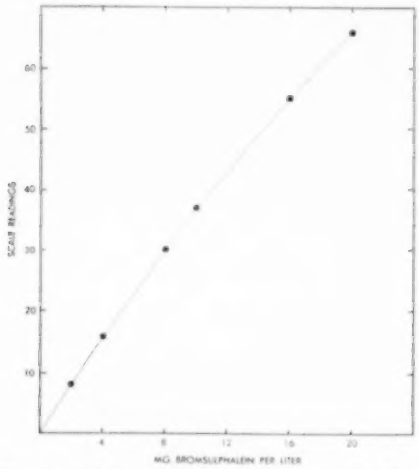

Fig. 1.—Relation of color intensity to alkaline solutions of Bromsulphalein.

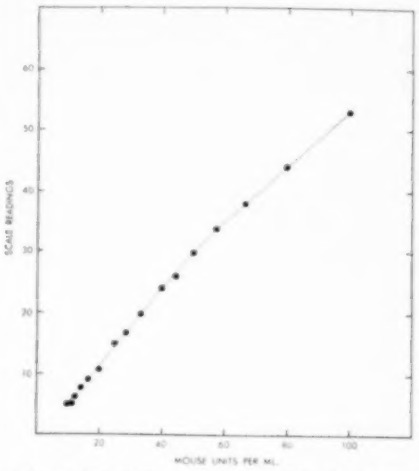

Fig. 2.—Relation of color intensity of alkaline solution of precipitate complex to number of mouse units per milliliter.

No. 525B (green) filter. The electrophotometer was adjusted to zero reading with a standard consisting of 2 ml. of saline and 2 ml. of indicator solution diluted with distilled water to a volume of 25 ml.

RESULTS

Figure 1 shows the standard Bromsulphalein curve obtained with known quantities of Bromsulphalein.

Various dilutions in 0.85% sodium chloride of Lot C-2 Special were made covering the range of 10 to 100 mouse units per ml. The color intensity of an alkaline solution of the precipitate complex obtained by the Bromsulphalein precipitation method was recorded. Figure 2 indicates the relation of color intensity to the toxic index of Lot C-2 Special venom dilutions of predetermined mouse units.

Each crude cobra venom and purified venom solution was assayed by mouse toxicity tests and the Bromsulphalein precipitation method. Mouse units by colorimetric titration were ascertained from Fig. 2. Table I summarizes these results.

SUMMARY

Use of an acid, buffered solution of Bromsulphalein has been extended to the titration of cobra venom. Titration values for six lots of cobra (Naja) venom by this colorimetric method were found proportional to the toxic indices in mice.

The proposed assay method is probably not for a specific fraction but for the titration of free amino groups and any other free radicals in the venom capable of binding the dye at acid *p*H. Although it does not obviate a toxicity test for a safety factor, a quantitative color test for certain chemical constituents in cobra venom is described which has been demonstrated useful for production control and preliminary assay of cobra venom solutions.

Table I.—Comparison of Toxic Index of Cobra Venom Determined by Colorimetric Titration and Mouse Toxicity Methods

Lot. No.	Mouse Units per Ml. Colorimetric Titration C^a	P^b	Mouse Toxicity, P
C-2 Special	..	100.0	100.0
A	64.0	56.5	60.0
B	64.0	54.2	55.5
C	64.8	55.5	60.0
D	62.9	55.6	55.5
E	64.0	56.0	60.0
F	62.9	54.5	55.5

[a] C = Crude venom.
[b] P = Purified venom.

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An Investigation of the Chronic Toxicity and Acceptability of Castrix*,†

By JAMES M. CRAMPTON‡ and ELBERT VOSS§

Investigations have been made of the effects of prolonged exposure of the albino rat to Castrix (2-chloro-4-dimethylamino-6-methylpyrimidine, and the acceptability of Castrix when administered to rats in their food and water. Results indicate a low degree of chronic toxicity. Reproductive ability is not reduced following administration of Castrix. A low degree of acceptability was demonstrated for Castrix-poisoned food and water.

THE CONTROL of rats by means of rodenticidal chemicals has always been complicated, not only by the difficulty of finding an efficient poison which will be taken by the rats, but also by the danger of accidental poisoning, particularly of children and domestic animals. In recent years several new rodenticidal agents have been investigated. Sodium fluoroacetate has been found to be highly efficient and probably the most satisfactory, except for the danger to other animals (1), but the lack of any known antidote has been a serious impediment to its use. Thus when DuBois (2) reported a new rodenticide, Castrix, which could be readily antidoted by sodium pentobarbital, and which he reported to be more toxic to rats than sodium fluoroacetate (3), it appeared that a major advance toward greater safety and efficiency in rodent control had been made. Literature reports on Castrix have not so far included any statements as to its chronic toxicity effects.

The rodenticidal activity of Castrix was first observed in Germany during World War II, and it is currently in use in that country for the extermination of field rodents. The drug used in this series of experiments was supplied by H. J. Spencer of the U. S. Department of Interior, Fish and Wildlife Service. This material consisted of a mixture of at least two isomers of Castrix and was yellowish brown in color. It differed in this respect from the Castrix in use in Germany which when pure is almost white. The compound has an acrid odor discernible in concentrations as low as 0.1 per cent in water. It dissolves slowly in water and difficulty is encountered in preparing aqueous solutions of more than 100 mg./L. It is readily soluble in alcohol.

When administered orally or parenterally to rats Castrix produces violent convulsions of a mixed clonic and tonic type. These convulsions have been described by DuBois (2). After several convulsions, separated by periods of grave depression, the rats may die. Rigor sets in within a few minutes. Following sublethal doses, convulsions and subsequent periods of depression are followed by apparently complete recovery.

Experiments were devised to indicate the effects of prolonged exposure to Castrix with reference to growth, kidney function, reproductive ability, and histologic pathology. Other experiments were undertaken to determine the acceptability of Castrix in food and water.

CHRONIC TOXICITY

In these experiments rats were housed in individual round cages and fed commercial food *ad libitum*. The Castrix was administered in all cases as a solution in water constituting the only source of drinking water. Two series were run. Series I was designed to show the effect of a maximum sublethal concentration on growth and histologic pathology. Series II was designed to show the effects of prolonged exposure on growth, kidney function, and reproductive ability. For series I, consisting of a group of eight rats, a concentration of 50 mg./L. was chosen since preliminary studies had indicated that about 50% mortality could be expected at this dosage level over a period of six weeks. For series II the concentration was reduced to 35 mg./L. so as to allow for the accumulation of a greater body of data by reducing the mortality rate.

Series I.—This series consisted of eight rats of equal age and weight. They were exposed to Castrix solution 50 mg./L. for a period of seven weeks. Tabulation of weights and Castrix consumption during the course of exposure (Table I) show that during the first four weeks the animals suffered a loss in weight, followed by a great increase from the fifth through the seventh week. A high mortality rate paralleled the period of loss in weight; however, the weight loss was no greater among the animals that died than among the survivors. There appears, moreover, to be no correlation between the dosage consumed and the mortality. In fact the average dose consumed by the surviving rats was greater than the dose consumed by the animals which died. This may be explained on the hypothesis that the total dose is of less importance than

* Received September 19, 1951, from the College of Pharmacy, University of Florida, Gainesville.

† Abstracted in part from a thesis submitted to the Graduate School of the University of Florida by James M. Crampton, in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

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TABLE I.—CASTRIX CONSUMPTION AND WEIGHT CHANGES AMONG RATS OF SERIES I

Week	Av. Wt., Gm.	Av. Daily Consumption Castrix Soln., Ml.	Av. Daily Dose, Mg./Kg.	No. Surviving at Beginning of Week
1	217	9.5	2.2	8
2	198	11.3	3.0	6
3	202	9.8	2.4	4
4	195	10.8	2.9	4
5	242	18.0	3.9	3
6	267	20.6	4.0	3
7	276	18.7	3.4	3

the dose consumed at one time. All the rats that died appeared to have succumbed to an acute fatal dose rather than to any chronic toxic effects.

Hemoglobin estimations on the three rats surviving the seventh week of intoxication were not significantly different from those of control rats which had been housed and fed under the same conditions. Surviving animals were then sacrificed for tissue studies, the results of which are reported below.

Series II.—This series consisted of 14 young female albino rats which were given Castrix solution, 35 mg./L., as their only source of drinking water for a period of thirty days. Ten rats from the same shipment were run as controls. Weight and water-Castrix solution consumed were recorded twice weekly. Metabolism cages were improvised by placing the individual round cages on racks fitted with funnels to collect the urine. Food was removed and twenty-four-hour urine samples were collected twice weekly.

Kidney Function.—Twenty-four-hour urine samples were collected twice weekly. The volume was recorded and tests for albumin and hemoglobin were run. Roberts' reagent was used in testing for albumin, and the benzidine hydrogen peroxide test was employed for hemoglobin. Urine volume for poisoned and control animals did not differ significantly. Albumin was found consistently in both control and poisoned animals. Semiquantitative methods for reporting based on the width and opacity of the line of flocculation failed to indicate any difference between controls and poisoned animals. Hematuria was not found. Microscopic examinations, which were done occasionally, failed to reveal anything of a pathological nature.

Reproduction.—The 12 rats remaining at the end of thirty days' poisoning with Castrix, 35 mg./L., were mated with normal males at a ratio of three females with one male. The control group of 10 female rats were also mated with normal males, three females with one male, and, for the tenth, one female with one male. Each group was maintained in a separate cage and disturbed only to replenish food and water supplied for ten days. The males were then removed. The females were kept under observation and as parturition appeared imminent were removed to individual cages to have their litters.

The males used above were then subjected to poisoning with Castrix solution, 35 mg./L., in their drinking water for periods of ten, twenty, and thirty days. They were then mated each with three normal females and in each case these males sired litters. Since no significant difference in fertility was noted in the animals poisoned for different periods of time they are all grouped together in Table III. The results of these experiments, summarized in Table III, indicate little if any reduction in fertility after Castrix poisoning. Litters were weaned when about three to four weeks old. There was no evidence of retardation of growth or development of the litters from poisoned parents.

TABLE II.—SUMMARY EXPERIMENTAL DATA OF SERIES II

Week	1	2	3	4
Av. daily dose, Mg. of Castrix/Kg.	3.35	3.60	3.77	3.85
Av. consumption of water, cc.				
(1) Poisoned	95.8	102.9	107.7	110.0
(2) Controls	120.1	130.8	162.4	170.1
Av. Wt., Gm.				
(1) Poisoned (initial wt. 140.1)	151.2	162.1	163.8	166.4
(2) Controls (initial wt. 139.6)	159.3	174.8	174.8	182.4

Effects on Growth.—As may be seen in Table II, the growth rate of the poisoned animals was depressed throughout the course of the experiment. This depression was, however, not very severe and ten days after withdrawal of the poison the animals had increased an average of 12.4 Gm., compared with 5.7 Gm. for the controls. This rapid increase following withdrawal of the Castrix would seem to indicate that the observed growth depression was due mainly to dehydration and nutritional reduction rather than to any organic changes. During this experiment none of the rats died, although two escaped from their cages and were not continued in the experiment. The lower mortality rate in series II is not due to a reduction in total dose, since the average daily dose for the rats in series II is actually higher than in series I. The low mortality in series II, in spite of high daily consumption, indicates a rapid detoxication or elimination of the poison. DuBois reported an LD_{50} of 1.25 mg./Kg. when administered orally to the albino rat (3).

HISTOLOGIC PATHOLOGY

A study of the histologic pathology resulting from chronic poisoning was made on five animals. Three of those studied were the survivors of the eight rats exposed to drinking water containing 50 mg. Castrix/L. One was the only survivor of four rats given intraperitoneal injections of 1.25 mg./Kg. three times a week for four weeks. Another was taken from a group of six rats exposed to drinking water containing 20 mg. Castrix/L. for seven weeks, none of which died. These animals were killed by severing the jugular veins. A gross examination of vis-

TABLE III.—FERTILITY OF RATS AFTER CASTRIX POISONING

	Female		Male	
	Control	Intoxicated	Control	Intoxicated
No. tested	10	12	8	7
No. proved fertile	9	8	8	7
Av. litter size	7.67	8.25	7.67	7.24
% Impregnation	...	...	90.00	81.00

ceral and reproductive organs was made and tissues were taken for microscopic study. These tissues were dehydrated, imbedded in paraffin, sectioned, and stained with hematoxylin and eosin, using standard techniques.

Gross examinations revealed no abnormalities. Microscopic examination of heart, kidney, pancreas, spleen, adrenal, intestine and ovary revealed no pathology. The lungs of three animals showed some inflammation with serous exudation and hemorrhaging into alveolar spaces. Necrosis of slight to moderate degree was present around the central veins of two of the five livers examined. These also exhibited congestion of central and portal veins. The testes of the three male rats were found in what appeared to be essentially a state of inactivity. Spermatozoa were absent and the tubules were small, irregularly scattered and atrophic. Germinal cells were greatly reduced in size and number, but a basal layer of spermatogonial cells was usually evident. Interstitial areas, while somewhat less conspicuous than normal, were nevertheless well differentiated and exhibited what appeared to be a slight reduction in size and number of individual cells.

The pronounced reduction of spermatogenic elements in the testes would indicate some depression of functional activity of these organs, if not a minor degree of actual damage. However, the preservation of the basal layer of spermatogonia and the interstitial elements provide the basis for prompt regeneration of sexual potency following withdrawal of the poison. Changes observed in lung and liver may indicate some damage to these organs, but it cannot be certainly ascribed to Castrix, and, in any case, the damage does not appear to have been sufficient to cause the death of any of the animals in these experiments. It is believed that in all cases death was due to exhaustion paralysis following a convulsive seizure. These convulsions usually resulted in bleeding from abrasions around the nose and mouth as a result of biting the wires of the cage during the seizure. Invariably dead rats removed from the cages showed these signs of having had a convulsion before death. These findings support our belief that all deaths were the result of ingestion of an acute fatal dose of the poison.

ACCEPTABILITY OF CASTRIX

In Water.—It has previously been reported that Castrix has a high degree of acceptability to rats (2). However, the reduced fluid consumption encountered in our work above led us to question the acceptability at least in aqueous solution. Since solutions of 50 mg./L. constituting the only source of drinking water had killed only 50–65% of the animals exposed and then only after periods of several days or weeks of exposure, it seemed evident that much higher concentrations would be required to give satisfactory results under field conditions. Therefore, a solution containing 200 mg./L. of water was prepared and its acceptability tested by giving rats an opportunity to choose between this solution and tap water. Twenty rats were placed in four cages and each cage supplied with two drinking bottles, one containing the Castrix solution and one containing tap water. The positions of the bottles were changed daily and the amounts of water and Castrix solution consumed were recorded for a period of ten days. The rats showed a decided preference for tap water. They drank an average of 2.3 ml. of Castrix solution per day per rat, compared with 20.4 ml. of tap water per day per rat. During this period of ten days 8 of the 20 rats died of Castrix poisoning. The 12 survivors were then given only Castrix solution for seven days. At the end of this time 5 of the 12 rats were still alive but greatly emaciated due to practically complete abstinence from water and food.

In Food.—Poisoned food containing 0.5% Castrix was also prepared by mixing the Castrix with the regular commercial food. This poisoned food was tested for acceptability on four groups of rats.

Group A.—Eleven rats were placed in a large cage and given only this poisoned food to eat. Four rats died the first day, four the second day, two the third day, and the last one on the fourth day. These rats consumed an average of 2.4 Gm. of poisoned food per rat per day.

Group B.—Five rats were starved for twenty-four hours, then given Castrix poisoned food. All were dead within twelve hours.

Group C.—Ten rats were placed in a large cage and given both poisoned and unpoisoned food to eat. This food was replenished as needed, and the amounts of each type consumed were recorded. These figures are not in themselves accurate since they include a considerable amount of wasted food which fell through the bottom of the cage. The ratio between the poisoned and unpoisoned food is, however, valid, since the amount of crumbs lost must be proportional to the amount of food eaten. It was found that the average daily consumption and wastage per rat per day of poisoned food was 0.27 Gm. as compared with a daily average of 14.8 Gm. of normal food over a ten-day period. None of these rats died.

Group D.—Seven rats were placed in a large cage and given approximately 100 Gm. of poisoned food and an equal amount of normal food. One rat died on the first day. By the end of the second day all the normal food had been consumed but most of the poisoned food remained. No more food was added. With only poisoned food remaining two rats died on the fourth day, one on the sixth, one on the eighth, and the last one on the eleventh day.

The results of these tests indicate a very low acceptability of food and water poisoned with the isomeric mixture used in this experiment. Aqueous solutions appear to be of little or no value as a rodenticidal agent. The poisoned food as prepared in this experiment also seems to be of no value as long as other sources of food are available. In this connection it is of interest to note that those rats, which had access to unpoisoned food when the Castrix poisoned food was first introduced (group D),

showed a greater aversion to the poisoned food than those which were given only Castrix-poisoned food at the beginning of the experiment (group A). It may be that by using some food which rats find highly palatable, the efficacy of this agent as a rodenticide could be increased. Indiscriminate use, without regard to the attractiveness of the diluent, however, would probably quickly condition the rat population to reject the poisoned bait. These conclusions apply only to the mixture of isomers of Castrix used in this experiment. Since the pure Castrix as used in Germany is readily acceptable and highly effective it appears that the presence of isomers greatly reduces the acceptability of the compound and hence its usefulness.

SUMMARY AND CONCLUSIONS

From the results of an investigation of the chronic toxicity of Castrix when administered to rats in their drinking water in concentrations of 50 and 35 mg./L., resulting in average daily doses of about 3 to 4 mg./Kg. for periods up to fifty days, it appears that there is little chronic toxicity. A moderate growth depression was observed, but this depression was rapidly overcome when the Castrix was withdrawn. No kidney damage was observed, and neither was reproductive ability impaired after withdrawal of the Castrix. Microscopic examination of tissues after varying periods of exposure revealed no damage to heart, pancreas, intestine, kidneys, adrenals, or ovaries. Slight to moderate damage was seen in two of five livers and three of five lungs. Testes appeared to be rendered inactive with marked reduction in spermatogenic elements. However, fertility tests indicate a rapid regeneration of sexual potency following Castrix withdrawal.

The low mortality associated with average daily doses of 3–4 mg./Kg., when compared with the previously reported LD_{50} of 1.25 mg./Kg. indicates rapid detoxification or elimination of this drug.

Experiments in which rats were allowed to choose between poisoned and unpoisoned food and water indicate a very low acceptability of this mixture of isomers of Castrix when offered to the albino rat.

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Gravimetric Determination of Histamines*

By MORTON SCHMALL, ERNEST G. WOLLISH, and JULIUS GALENDER†

The method presented is based upon the gravimetric determination of histamine as its nitranilate. It is suitable for the assay of histamine and its salts in pure form as well as in pharmaceutical preparations, such as ointments, parenteral solutions, and tablets. An extraction technique for the separation of histamine dihydrochloride from pharmaceutical ointments is described.

SEVERAL colorimetric methods for the determination of histamine in pharmaceutical preparations are reported in the literature. They are based upon the interaction between histamine and a suitable coupling agent. Mader, *et al.* (1) used 1,2-naphthoquinone-4-sodium sulfonate; McIntire, *et al.* (2) reacted histamine with 2,4-dinitrofluorobenzene, while other workers used diazotized aromatic amines to develop the color (3–8).

Although these methods are useful for the determination of small quantities of histamine and its salts, a method for the assay of histamine salts in pure form as well as in pharmaceutical preparations, applying macrotechniques, seemed desirable.

Towne (9) precipitated glycine from a mixture of glycine and alanine with nitranilic acid, the preparation of which is described by Meyer (10), while Block (11) used this reagent to precipitate histidine quantitatively from either an aqueous, acetone, or alcoholic medium. We found that histamine could also be quantitatively precipitated as its nitranilate under controlled conditions in pure form as well as in pharmaceutical preparations. Histamine combines with one

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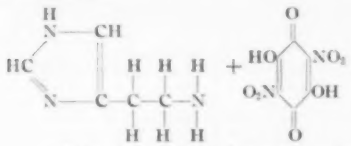
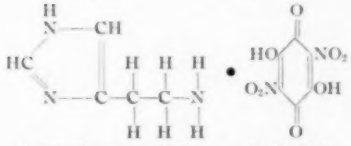
mole of nitranilic acid to give the insoluble histamine nitranilate.



Histamine ↓ Nitranilic acid



Histamine nitranilate $C_{14}H_{11}N_5O_8$

Molecular weight 341.23

An elementary microanalysis of the precipitate gave the following results:

Anal.—Calcd. for C, 38.71%; H, 3.25%; N, 20.53%. Found: C, 38.88%; H, 3.33%; N, 20.31%.

EXPERIMENTAL

Assay of Histamine Dihydrochloride or Phosphate

Reagents.—

(*a*) A filtered 3.5% solution of nitranilic acid in 95% alcohol.

(*b*) Ethyl ether.

(*c*) 95% alcohol.

(*d*) Chloroform-petroleum ether mixture. Mix 1 part petroleum ether, b. p. 35–70°, and 4 parts chloroform.

Procedure

A sample of approximately 100 mg. of histamine dihydrochloride or about 150 mg. of histamine phosphate is weighed accurately into a 100-cc. beaker. After dissolving the sample in 10 cc. of water, 10 cc. of nitranilic acid reagent is added slowly with constant stirring. The mixture is allowed to stand for about fifteen minutes, then 10 cc. of 95% ethanol is added while stirring. The mixture is then allowed to stand i the refrigerator for three hours. The precipitate is filtered through a tared 30-cc. sintered glass crucible of medium porosity. The beaker and precipitate are washed with four 5-cc. portions of cold ethanol (about 10°). Finally it is washed with 10 cc. of ethyl ether. The precipitate is dried in an oven at 120° for two hours, cooled in a desiccator, and weighed. Each milligram of the precipitate is equivalent to 0.5394 mg. of histamine dihydrochloride, or 0.9009 mg. of histamine phosphate.

Assay of Histamine in Ointments

A sample of ointment containing approximately 100 mg. of histamine dihydrochloride or 150 mg. of histamine phosphate is accurately weighed into a 50-cc. centrifuge tube. Twenty to twenty-five cubic centimeters of the chloroform-petroleum ether mixture is added and the tube is immersed in a water bath at 55–60° for five minutes. Ten cubic centimeters of water is added. While keeping the temperature at 55–60°, the mixture is thoroughly agitated for two minutes, using an electric stirrer. The stirring rod is washed with a small portion of water and the tube centrifuged for about thirty seconds at 2,500 r. p. m. Most of the aqueous layer is carefully drawn off with a 20-cc. hypodermic syringe, fitted with a No. 13 three-inch needle with a blunt point. The aqueous solution is filtered through a Whatman No. 2 filter paper of 11 cm. diameter into a 100-cc. volumetric flask. The syringe is rinsed with about 5 cc. of water which is added through the filter to the filtrate. The centrifuge tube is again immersed in the warm water bath. Ten cubic centimeters of water is added and the extraction and filtration repeated three more times. The filtrate is brought to room temperature and made up to 100 cc. with water. Since histamine nitranilate has no sharp melting point, the following identity test could be applied to an aliquot of the solution:

Identity Test.—A 5-cc. aliquot is pipetted into a small beaker to which 15 cc. of a filtered saturated aqueous solution of picric acid is added. The precipitate is filtered and washed with a little water and dried at 150° for one hour. The melting point of histamine mono-picrate is 233–234° (corr.).

The remaining 95 cc. in the volumetric flask is quantitatively transferred to a 250-cc. beaker, using water to rinse the flask. The solution is then evaporated on a steam bath to about 10 cc. with the aid of a jet of air to hasten evaporation. The procedure is then continued as previously described for pure histamine salts.

Results.—Results obtained by repeated determinations on samples of commercial histamine dihydrochloride and phosphate are listed in Table I.

Results obtained on pharmaceutical preparations containing histamine salts are given in Table II.

It was found that a reproducibility of ±1.0% with pure compounds and ±2.0% on samples of pharmaceutical preparations could be obtained, as is apparent from the results in Tables I and II.

DISCUSSION

When assaying samples of histamine phosphate, it is important that during the precipitation of the nitranilate the quantity of alcohol present does not exceed 60%. When alcohol was added dropwise with stirring to a solution containing 150 mg. of histamine phosphate in 10 cc. of water, the histamine phosphate began to precipitate after the addition of 32 cc. of alcohol. The method as described, requiring addition of 10 cc. of alcoholic nitranilic acid, is therefore within the limits of solubility of the histamine phosphate in an aqueous alcoholic medium.

Some of the constituents that may be found in ointments like isopropylamine, di- and tri-ethanolamine, methyl salicylate, menthol, and lanolin gave no precipitate with nitranilic acid. Glycine and histidine, which resulted in precipitates, can be identified by a modification of the ninhydrin color test (12), carried out in the following manner: To 5 cc. of an aqueous solution of the sample, 10 drops of a 1% aqueous solution of ninhydrin is added and the mixture is heated on a boiling water bath for two minutes. A blue color indicates the presence of histidine or glycine.

TABLE I

Sample	Weight of Sample, Gm.	Weight of Precipitate, Gm.	Assay, %	Deviation from Mean
Histamine dihydrochloride, Detn. No.				
1	0.1011	0.1865	99.5	+0.3
2	0.1109	0.2037	99.1	−0.1
3	0.1205	0.2218	99.3	+0.1
4	0.1290	0.2360	98.7	−0.5
5	0.1521	0.2797	99.2	0.0
		Mean:	99.2	
Histamine phosphate, Detn. No.				
1	0.1501	0.1644	98.7	−0.1
2	0.1511	0.1645	98.1	−0.7
3	0.1500	0.1655	99.4	+0.6
4	0.1493	0.1641	99.0	+0.2
		Mean:	98.8	

TABLE II

Type of Preparations	Quantity of Sample Used	Claim	Weight of Precipitate, Gm.	Assay, %
Ointment				
Sample A				
Detn. No. 1	10 Gm.	1% (as dihydrochloride)	0.1836	0.99
Detn. No. 2	10 Gm.	....	0.1817	0.98
Detn. No. 3	10 Gm.	....	0.1873	1.01
Detn. No. 4	10 Gm.	....	0.1854	1.00
Sample B	10 Gm.	....	0.1815	0.98
Sample C	10 Gm.	....	0.1857	1.00
Sample D	10 Gm.	....	0.1870	1.01
Sample E	10 Gm.	....	0.1869	1.01
Sample F	10 Gm.	....	0.1860	1.00
Hypodermic tablets				
Detn. No. 1	25 tablets	3.0 mg./tab. (as phosphate)	0.0852	3.07 mg./tab.
Detn. No. 2	25 tablets		0.0860	3.10 mg./tab.
Ampuls				
Detn. No. 1	15.0 cc.	2.75 mg./cc. (as phosphate)	0.0476	2.86 mg./cc.
Detn. No. 2	15.0 cc.		0.0466	2.80 mg./cc.

If the *p*H of an ointment is above 7.0, the histamine may be present as the free base, and care should be taken that none of the histamine is lost during the evaporation. In this case, 1 cc. of dilute hydrochloric acid is added to the aqueous solution, which is then evaporated to dryness. The residue is dissolved in 10 cc. of water and the procedure continued as described.

SUMMARY

A gravimetric method for the assay of histamine phosphate and dihydrochloride is described. It is based upon the quantitative precipitation of histamine as nitranilate. The method can also conveniently be used for the determination of histamine salts in pharmaceutical preparations. An extraction technique for water-soluble histamine salts from ointments is described. A precision of ±1.0 per cent on pure salts and ±2.0 per cent on pharmaceutical preparations was obtained.

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Studies of Lactic Acid Producing Organisms in a Microbiological Assay of Penicillin*,†

By MURIEL C. VINCENT, EDWARD KRUPSKI,‡ and LOUIS FISCHER§

Five nonpathogenic organisms were investigated to determine their applicability to a quantitative assay of penicillin. Various media were investigated to determine the optimum growth conditions for the organisms as evidenced by the amount of acid produced at several *p*H levels with different periods of incubation. The effects of numerous growth factors upon the acid production of the organisms were determined. The effects of increasing concentrations of penicillin on the growth of the various organisms were observed. It was found that *Lactobacillus casei* produced a high initial acid concentration with marked decreases at successively higher levels of penicillin concentration, indicating that this organism could be applied to a microbiological assay of penicillin.

MICROBIOLOGICAL METHODS of penicillin assay are based on the fact that the amount of penicillin quantitatively affects the degree of growth of the test organisms. The inhibition of lactic acid production of *Streptococcus fecalis DK* by penicillin was utilized by Sager and Arrigoni (1) in their assay procedure for the antibiotic.

Since a number of microorganisms produce acid as a metabolic product, a fact utilized in several microbiological assays, experiments were undertaken to determine whether the use of different nonpathogenic acid-producing microorganisms, the addition of various growth factors, change in *p*H, or variation of the medium would improve the results of the assay procedure as reported by Sager and Arrigoni.

EXPERIMENTAL

The experimental work of this investigation was divided into three parts. The first consisted of determining the optimum conditions of growth for various organisms. The second involved determining the effect of certain growth factors on the acid production of the organisms grown under the optimum conditions, and the third, the effect of penicillin, at different concentrations, on the growth of the organisms.

Organisms Used.—The five lactic-acid producing organisms selected were: *Lactobacillus bulgaricus* A. T. C. C. No. 7993, *Lactobacillus bulgaricus* A. T. C. C. No. 9224, *Lactobacillus casei* A. T. C. C. No. 7469, *Lactobacillus thermophilus* A. T. C. C. No. 8317, and *Streptococcus thermophilus* A. T. C. C. No. 7952.

Media Used.—A number of media were used to determine optimum conditions for growth of the organisms. These included the Sager and Arrigoni medium, which will be designated as medium A; medium A with 0.6% lactose; riboflavin assay medium (2); niacin assay medium (2); Difco lactose broth (1.3%); Difco peptone broth (2%); Difco peptone (1%) and dextrose (2%) broth; Difco Lyptone (1%), Armour beef extract (2%), and lactose (2%) broth; and Difco peptone (1%), powdered skim milk (6.25%), and lactose (0.5%) broth.

Determination of Optimum Conditions for Growth of Organisms.—The criterion employed to indicate optimum growth conditions for the organisms was the amount of lactic acid produced. Portions of 10 ml. of the media were inoculated with the organism, and after incubation, the acid produced at the different *p*H levels was determined by titrating with 0.05 *N* sodium hydroxide at twelve-hour intervals for a seventy-two-hour period. A Beckman *p*H meter was used to determine the end point. All organisms were incubated at 37° except *Lactobacillus thermophilus* No. 8317 and *Streptococcus thermophilus* No. 7952, which were incubated at 55° and 47°, respectively.

The optimum conditions found are recorded in Table I.

Effects of Growth Factors.—Various growth factors were employed to determine their effect on the organisms under investigation. On the basis of reports in the literature, at least four concentrations of each growth factor were used. These substances and the concentrations of each investigated are listed in Table II.

The media and *p*H used were those which caused the organism to produce the greatest amount of acid. Portions of 10 ml. of the media containing the growth factor were inoculated with the organism and incubated for the optimum period of time. The increase or decrease in acid production was measured by titration with 0.05 *N* sodium hydroxide and comparison with a control containing no growth factor.

The only pronounced increase in acid production was shown by *Lactobacillus casei* No. 7469 in the A medium, *p*H 7, to which 3.0 units of liver extract and 0.75 μg. of vitamin B_{12} were added; other concentrations of these factors were less effective. The other organisms were not affected to any significant degree by the factors studied.

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† Abstracted in part from a thesis presented by Muriel C. Vincent in partial fulfillment of the requirements for the degree of Master of Science in Pharmacy.

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TABLE I.—CONDITIONS SELECTED FOR THE VARIOUS ORGANISMS

Organism	Media	*p*H	Incubation Period, Hr.
Lactobacillus bulgaricus No. 7993	A	7.8	72
	A-lactose	7	72
	Peptone-milk-lactose	6.4	72
Lactobacillus bulgaricus No. 9224	A	9	72
	Peptone-milk-lactose	6.4	72
Lactobacillus casei No. 7469	A	7	72
	Riboflavin	7	72
Lactobacillus thermophilus No. 8317	A-lactose	8	72
Streptococcus thermophilus No. 7952	A	8	72

Effect of Penicillin on Growth of Organisms under Investigation.—The reaction of the various organisms to different concentrations of penicillin was observed under the optimum conditions, found in the preceding experiments, in order to evaluate their applicability to a quantitative assay of the antibiotic.

With minor modifications the general procedure of Sager and Arrigoni (1) was applicable to the five organisms under investigation. The conditions used were those listed in Table I with one exception, the A medium was fortified with liver extract and vitamin B_{12} when used with *Lactobacillus casei* No. 7469.

The strength of the standard penicillin solution was such that both the growth of the organisms and the inhibition by penicillin could be observed.

Three series of penicillin concentrations were used, and one or more of these were employed with each organism. The series contained 0, 1, 2, 3, 4, and 5 units; 0, 4, 8, 12, 16, and 20 units; and 0, 8, 16, 24, 32, and 40 units of penicillin per assay tube.

Lactobacillus bulgaricus No. 7993, *Lactobacillus bulgaricus* No. 9224, *Lactobacillus thermophilus* No. 8317, and *Streptococcus thermophilus* No. 7952 showed small or inconsistent decreases in acid production on addition of penicillin. The large amount of acid produced by *Lactobacillus casei* No. 7469 in the fortified A medium and the inhibition of the organism by penicillin, as demonstrated by the marked decrease in acid production, were distinguishing factors that suggest the use of this organism as an improvement of the penicillin assay method suggested by Sager and Arrigoni. With this organism a pronounced reduction of acid production at the various penicillin concentrations was observed, as is shown in Fig. 1.

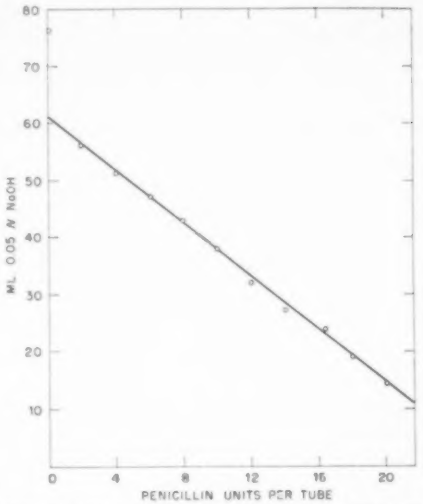

Fig. 1.—Acid production inhibition of *L. casei* by penicillin.

Commercial samples of penicillin were assayed according to this method and satisfactory results were obtained.

SUMMARY

Five nonpathogenic lactic-acid producing organisms were investigated to determine their applicability to a quantitative assay of penicillin.

Various media were investigated to determine the optimum growth conditions for the organisms as evidenced by the amount of acid produced at several *p*H levels with different periods of incubation.

TABLE II.—CONCENTRATIONS OF GROWTH FACTORS

Growth Factor	Concentrations per 10 Ml. of Media			
Adenine sulfate	25	50	75	100 μg.
Adenine sulfate–guanine hydrochloride–uracil mixture (equal parts of each)	0.2	0.4	0.5	0.8 mg.
Biotin	0.0015	0.0025	0.005	0.01 μg.
Guanine hydrochloride	25	50	75	100 μg.
Liver extract	0.75	1.5	3.0	4.5 units
Liver extract—vitamin B_{12}	1.5 0.75	1.5 1.125	3.0 0.75	1.5 units 1.5 μg.
Liver protein hydrolysate	0.5	1	2	3 mg.
para-Aminobenzoic acid	5	10	15	20 μg.
Phenylacetic acid	25	50	75	100 μg.
Pyridoxine hydrochloride	5	10	15	20 μg.
Vitamin B_{12}	0.15	0.75	1.5	3.0 μg.

The effect of several growth factors upon the acid production of the organisms was determined. No substantial increase was noted except in the case of liver extract-vitamin B_{12} with *Lactobacillus casei* A. T. C. C. No. 7469.

The effect of increasing concentrations of penicillin on the growth of the various organisms was also studied.

Lactobacillus casei A. T. C. C. No. 7469 is suggested as an organism to replace *Streptococcus fecalis DK* as recommended by Sager and Arrigoni. In the use of this organism greater amounts of acid were produced with more significant decreases on the addition of increasing amounts of penicillin, thus leading to greater accuracy of the method.

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Optical Crystallographic Properties of Organic Compounds. III. Esters of *p*-Hydroxybenzoic Acid*,†

By RAYMOND N. CASTLE‡

The following optical crystallographic data for five esters of *p*-hydroxybenzoic acid have been determined: The principal indices of refraction, α, β, and γ; the birefringence, B; the optic sign; the type and extent of dispersion; elongation; habit; crystal system; extinction; and the $\sqrt{\alpha\beta\gamma}$. The size of the optical axial angle, $2V$, has been calculated from the indices of refraction, α, β, and γ. The optical orientation has been noted when constant. The optical crystallographic constants allow rapid identification of the esters of *p*-hydroxybenzoic acid. The methyl ester can be readily recognized by the extremely strong anomalous dispersion and the small optic axial angle. The benzyl ester can be distinguished from the rest of the group by the positive optical character. The other three esters can also be rapidly identified by a combination of constants.

THE PURPOSE of this communication is to place on record the optical crystallographic properties of some of the esters of *p*-hydroxybenzoic acid which are used as preservatives in some foods, pharmaceutical preparations, and cosmetics. In the present communication the optical crystallographic properties of five esters are reported. They are: methyl *p*-hydroxybenzoate, ethyl *p*-hydroxybenzoate, *n*-propyl *p*-hydroxybenzoate, *n*-butyl *p*-hydroxybenzoate, and benzyl *p*-hydroxybenzoate.

The optical and crystallographic properties have been determined as completely as the available equipment and the crystal habit of the compound in question would allow. The principal indices of refraction, α, β, and γ, have been determined by the immersion method using interference figures as a guide in selecting properly oriented crystals. In some instances it was necessary to determine the α and γ indices of refraction from crushed fragments showing the highest polarization colors between crossed nicols due to the fact that centered acute bisectrix, centered obtuse bisectrix, or centered optic normal interference figures were not usually readily observed. These values of n will correspond to the principal indices of refraction when care is taken to insure random orientation of the crushed crystal fragment. The immersion technique using the Becke test has been thoroughly described in several excellent textbooks (1–4). The indices of refraction have been determined with an accuracy of ± 0.002 except where otherwise noted. When working at a temperature other than $25 \pm 1°$, temperature corrections were made. In certain instances in which the crystal was soluble in the immersion

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medium to an appreciable extent, the index of refraction of the saturated medium was determined at the temperature at which the index of refraction of the crystal was determined. Thus the comparison is absolute. For measurement of the indices of refraction the light from the sodium vapor lamp was used. This light contains the wave lengths of 5,889 and 5,895 Å. An average value for the indices of refraction was obtained from the cube root of the product of α, β, and γ, ($\sqrt[3]{\alpha\beta\gamma}$). The source of illumination for the other optical and crystallographic properties was a Spencer Microscope Lamp Model 370-B equipped with a Corning Daylite Filter. The indices of refraction were checked using daylight in order to determine dispersion of the indices of refraction.

The value of the optical axial angle, $2V$, was calculated from the values of the indices of refraction α, β, and γ, using the more exact formula suggested by Larsen and Berman (5).

The other optical and crystallographic properties are given in the experimental part and are discussed there.

The melting points were all determined in a stirred bath with the Anschutz thermometers at total immersion; thus no stem correction was necessary. All compounds were recrystallized from hot aqueous ethanol by allowing the solution to cool undisturbed.

EXPERIMENTAL

Methyl *p*-Hydroxybenzoate.—The crystalline compound was dried in a vacuum desiccator over phosphorus pentoxide, m. p. 125.8 to 127.0°. Well-formed crystals for the determination of crystal habit were obtained by crystallization from aqueous ethanol on a microscope slide.

Habit, Acicular to Equant.—This compound was assigned to the orthorhombic system (?) on the basis of the parallel extinction observed and upon the appearance of the crystals grown upon the microscope slide. Elongation was parallel to the slow vibration direction in the crystal, therefore positive. $2V = 36°$, calculated. The dispersion of the optic axis is extremely great, so much so that the black isogyres are frequently obscured by the intense red and green color fringes, $r > v$. The dispersion was unusual in that when the isogyres of the acute bisectrix interference figure were in the form of a cross (extinction position) the blue color fringes were all on one side of the black bar and the red or yellow color fringes were all on the other side. This at first appeared to be the horizontal dispersion of the monoclinic system except that, instead of the color fringes being parallel to the axial plane, they were at right angles to it. No dispersion of this type has ever been observed in the author's experience. XX' is the acute bisectrix, optic sign, negative. The commonly observed orientation gives an inclined acute bisectrix interference figure from which no principal indices of refraction were obtained. The extinction is incomplete on crystals showing the inclined acute bisectric interference figure. $\alpha = 1.590$, $\beta = 1.688$, $\gamma = 1.695$; β and γ were determined from centered acute bisectrix interference figures. $\sqrt[3]{\alpha\beta\gamma} = 1.657$. The birefringence, $B = 10.5 \times 10^{-3}$.

Ethyl *p*-Hydroxybenzoate.—The compound was dried in a vacuum desiccator over phosphorus pentoxide, m. p. 114.6 to 115.6°.

Habit, Tabular to Equant.—When crystallized on a microscope slide from aqueous ethanol the crystals are beautifully formed and show well developed prism faces. Crystals with prism faces are common and show a centered acute bisectrix interference figure from which the β and γ indices of refraction were obtained. Both acute bisectrix and optic axis interference figures are shown on fusion preparations. This compound was assigned to the orthorhombic system on the basis of the parallel extinction observed and upon crystal morphology. Elongation is parallel to YY', therefore elongation is either positive or negative depending upon the orientation of the crystal. Reasonably strong dispersion of the optic axis, $v > r$. XX' is the acute bisectrix optic sign, negative. $2V = 64°$, calculated. $\alpha = 1.423$, $\beta = 1.648$, $\gamma = 1.768 \pm 0.003$. All three of the indices of refraction are dispersed in white light. $\sqrt[3]{\alpha\beta\gamma} = 1.606$; the birefringence, $B = 34.5 \times 10^{-3}$.

***n*-Propyl *p*-Hydroxybenzoate.**—The crystalline compound was dried in a vacuum desiccator over phosphorus pentoxide, m. p. 96.0 to 97.4°. Well-formed crystals were very difficult to obtain. When crystallization was attempted on the microscope slide from a variety of solvents, beautiful, feathery, radiating crystals were obtained. These, while diagnostic, were not useful for the determination of crystal system. A few lath-shaped crystals with pointed ends were obtained by crystallization from benzene on the microscope slide. These showed parallel extinction and on this basis were assigned to the orthorhombic system. The crystals were elongated parallel to the slow vibration direction, therefore the sign of elongation is positive. $2V = 70°$, calculated. Dispersion of the optic axes, $v > r$. XX' is the acute bisectrix, optic sign, negative. $\alpha = 1.488$, $\beta = 1.650$, $\gamma = 1.768 \pm 0.003$. The β index of refraction shows dispersion in white light. $\sqrt[3]{\alpha\beta\gamma} = 1.631$. The birefringence, $B = 28.0 \times 10^{-3}$.

***n*-Butyl *p*-Hydroxybenzoate.**—Crystals obtained from aqueous ethanol were very large and had to be crushed for microscopic study. When cooled rapidly from a variety of solvents the compound frequently separated as an oil which produced opaque crystals. These were not suitable for optical study. The crystals were dried in a vacuum desiccator over phosphorus pentoxide, m. p. 68.8 to 70.8°. When crystallized on a microscope slide, large masses of very fine crystals were obtained which were not suitable for optical study, thus the optical properties were determined on crushed fragments of the large crystals.

Habit, Tabular to Equant.—This crystal was placed in the orthorhombic system (?) on the basis of the parallel extinction exhibited on the few crystals which have a regular form. $2V = 61°$, calculated, strong dispersion of the optic axes, $v > r$. XX' is

TABLE I.—OPTICAL CONSTANTS OF THE ESTERS OF *p*-HYDROXYBENZOIC ACID

Ester of *p*-Hydroxybenzoic Acid	Indices of Refraction α	β	γ	Birefringence $(\gamma - \alpha)$	Dispersion	Optic Sign	$2V$	$\sqrt[3]{\alpha\beta\gamma}$
n-Butyl	1.436	1.645	1.744	30.8×10^{-3}	Strong axial, $v > r$	−	61°	1.603
Ethyl	1.423	1.648	1.768	34.5×10^{-3}	Reasonably strong axial, $v > r$	−	64°	1.606
n-Propyl	1.488	1.650	1.768	28.0×10^{-3}	Axial, $v > r$	−	70°	1.631
Benzyl	1.588	1.651	1.783	19.5×10^{-3}	Axial, $r > v$	+	82°	1.672
Methyl	1.590	1.688	1.695	10.5×10^{-3}	Extremely great, $r > v$[a]	−	36°	1.657

[a] Anomalous dispersion.

the acute bisectrix, optic sign, negative. Centered or nearly centered acute bisectrix interference figures were commonly observed on crushed fragments. From the centered acute bisectrix interference figures the β and γ indices of refraction were determined. $\alpha = 1.436$, $\beta = 1.645 \pm 0.003$, $\gamma = 1.744 \pm 0.003$. In white light the β index of refraction is dispersed. $\sqrt[3]{\alpha\beta\gamma} = 1.603$. The birefringence, $B = 30.8 \times 10^{-3}$.

Benzyl *p*-Hydroxybenzoate.—The compound was dried in a vacuum desiccator over phosphorus pentoxide, m. p. 109.0 to 111.0°. The compound when produced from aqueous ethanol was nicely crystalline and suitable for optical study. Beautiful acicular and lath-shaped needles were obtained when crystallized on a microscope slide from aqueous ethanol. Crystal system, orthorhombic, based on the parallel extinction observed and upon the morphology of the crystals. Elongation is parallel to YY', therefore both positive and negative depending upon the orientation of the individual crystal. $2V = 82°$, calculated, dispersion of the optic axes, $r > v$. ZZ' is the acute bisectrix, optically positive. The common orientation shows an inclined acute bisectrix interference figure from which the β index of refraction can be obtained. $\alpha = 1.588$, $\beta = 1.651$, $\gamma = 1.783 \pm 0.003$. All three indices of refraction are dispersed in white light. $\sqrt[3]{\alpha\beta\gamma} = 1.672$. Birefringence, $B = 19.5 \times 10^{-3}$.

DISCUSSION

The optical and crystallographic properties are useful physical constants that can be used for rapid identification of crystalline chemical compounds. The most significant constants from a determinative point of view are the principal indices of refraction. The birefringence, $B\ (\gamma - \alpha)$ is another important physical constant that can frequently be estimated rapidly. This is particularly true of organic compounds because of the more random cleavage. This random cleavage also aids materially in the ease of determination of the principal indices of refraction. The type and extent of dispersion are of considerable diagnostic value in organic compounds where examples of strong dispersion of various types are relatively common.

In Table I are listed the optical properties of the greatest value from a determinative point of view. They are listed in order of increasing value of the β index of refraction. In this particular series of compounds the values of the β indices of refraction lie very close together and as a result more than one index would have to be determined for positive identification. In most instances the α index of refraction could be used in this series as a diagnostic index if this is coupled with the dispersion characteristics of the crystal. With practice the optical axial angle can be estimated to within a few degrees from the curvature of the isogyre.

The identification of the methyl ester official in the U. S. P. as methylparaben can be accomplished in a very few minutes in contrast to the time-consuming official identification. The methyl ester is perhaps the easiest of the series to identify because of the very strong and unusual dispersion and because of the small axial angle. The *n*-propyl ester also official in the U. S. P. as propylparaben can be identified by use of the optical properties in a fraction of the time required for the official identification procedure. The other esters described in this investigation can be identified as rapidly as the *n*-propyl ester by use of the optical properties. The real advantage in the use of the optical properties for identification purposes is that crystal fragments can nearly always be used. This is in strong contrast to the use of crystal morphology as the criterion of identification. It is frequently difficult to obtain well formed crystals when they may have been isolated from a pharmaceutical preparation unless considerable time is spent in purification.

SUMMARY

The optical crystallographic properties of five esters of *p*-hydroxybenzoic acid have been determined; namely, methyl *p*-hydroxybenzoate, ethyl *p*-hydroxybenzoate, *n*-propyl *p*-hydroxybenzoate, *n*-butyl *p*-hydroxybenzoate, and benzyl *p*-hydroxybenzoate. The use of these physical constants has been suggested for purposes of identification of this series of esters which are employed industrially as preservatives.

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Digitalis Extraction Studies. III[*,†]

By RAYMOND E. HOPPONEN[‡] and OLE GISVOLD[§]

Crystalline glycosides have been isolated from the fresh leaves of *Digitalis lanata* and separated into two fractions by a modification of the Bay and Gisvold procedure. The higher melting fraction was hydrolyzed and digoxigenin, digitoxose, and acetic acid identified in the hydrolysis mixture. The higher melting fraction was de-esterified, resulting in a product identified as digoxin. This and other evidence presented indicates an acetyl digoxin. The second, lower melting fraction has been tentatively identified as *beta*-acetyl digoxin. Color reactions also indicate the presence of a gitoxigenin-containing component.

IN SEEKING a means for the preparation of stable solutions of the active principles of Digitalis comparatively free from extraneous materials, Bay and Gisvold (1) developed a procedure utilizing a water extract of the finely disintegrated fresh leaves. The leaves were disintegrated in a Waring blendor in the presence of a small amount of water. The mixture was then heated to coagulate the chlorophyll-carrying proteins and filtered. The filtrate obtained from the mixture was concentrated and saturated with sodium sulfate to precipitate the glycosides along with other plant materials on a filter cell. This precipitate served as the basis for the preparation of physiologically active solutions. King and Gisvold (2), in an extension of this work, showed that maximum extraction was obtained if the aqueous extract represented a 2 per cent solution of the drug calculated on the weight of the dried leaf. Their results, based on activity toward the frog, indicated that at least 87 per cent of the activity could be extracted in this way. It was found also that direct extraction of the saturated sodium sulfate solution with tetrahydrofuran was effective in recovering the active principles. Gisvold has shown (unpublished work) that active material can be obtained by direct extraction of the aqueous extract with methyl isobutyl ketone.

The object of this study was to attempt a separation and purification of the active materials extracted by this procedure in order to determine their nature; whether they were obtained as the "genuine" glycosides described by Stoll and Kreis (3) or as the long-known digitoxin and gitoxin types. *Digitalis lanata* was selected as the species for study since the content of glycosides is high and the "genuine" glycosides are crystalline and therefore would offer fewer difficulties in isolation.

EXPERIMENTAL

Grinding and Extraction.—Since the use of the Waring blendor in the application of the Bay and Gisvold procedure permitted operation on only a small scale, it was necessary to develop a modified method for disintegrating the large quantities of leaves needed for the isolation work. Freezing of the fresh leaves and powdering of the frozen material appeared to be a feasible procedure. This should also prevent or markedly retard any enzymatic action during the grinding process. The reports of Hepler, Ackerman, and French (4) and King and Gisvold (2) have pointed out that glycoside action appears unaffected by freezing. Accordingly, the following procedure was developed: mature leaves were frozen immediately after picking by packing into a wooden tub in alternating layers with crushed dry ice. Freezing was almost instantaneous, but to insure completeness, the packed tub was allowed to stand for twenty or thirty minutes before grinding. The mass was powdered in a Jacobson laboratory mill, the construction of which reduces contact time of material and machine to a minimum. Three powderings of each collection were effected, once through a 2/64 in. screen and twice through a $1^1/_2$/64 in. screen. The result was a "snow" of powdered leaves and dry ice which could be processed at once or stored in a frozen state for future use. The plant material used was obtained from plants grown in the Medicinal Plant Garden during the summer of 1949 by the Department of Pharmacognosy at the University of Minnesota. Harvesting was begun October 4 and continued through November 15. Determinations of the moisture content of the leaves were made at intervals during the harvesting period to provide a dry weight basis for the extraction work. Samples of 75–100 Gm. which were dried at 45° for forty-five hours showed an average moisture content of 77%.

After removal of the carbon dioxide, the still-frozen leaf material was dropped in portions with stirring into hot water (2.5 to 3.0 L. for each Kg. of leaf) maintained at a temperature of 65–70°. When addition was complete and the temperature reached 70°, the mixture was filtered through muslin into a graduated filter flask. The leaves were washed with sufficient hot water (70°) to bring the volume of filtrate to 3.0 to 3.5 L., this being the largest volume which could conveniently be handled. The marc

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was sucked dry, then resuspended in hot water and filtered again. The procedure was repeated as many times as necessary to obtain a total volume of filtrate which would represent a 2% solution of the leaves on a dry weight basis. The still-warm filtrates, which had a clear amber color, were extracted immediately with methyl isobutyl ketone. Three extractions were made of each portion of filtrate. The Keller test indicated that the major portion of the active material was removed by the first two extractions. Emulsions which formed in the ketone layer were readily cracked by warming on the steam bath. The filtered ketone extracts were concentrated at reduced pressure using a bath temperature of not over 60°. The resulting concentrate had a dark green color. On being allowed to evaporate at room temperature, it deposited a light-tan colored crystalline material which was removed by filtration and washed twice with methy isobutyl ketone. The air-dried material melted with decomposition at 180°. The Keller test showed a red-brown ring with a blue color in the acetic acid layer. Saturation of the aqueous filtrate with sodium sulfate after extraction with methyl isobutyl ketone caused the separation of some solid material. The mixture was shaken with methyl isobutyl ketone as before, but no material giving a positive Keller reaction was obtained. The solid material was separated, dried, and extracted with methyl ethyl ketone, one of the solvents used by King and Gisvold (2). This extract also contained no material indicative of glycosides. It appeared that all glycosidic material could be removed from the aqueous filtrate by direct extraction with methyl isobutyl ketone.

The mother liquors of the concentrate from which the crystalline material had separated were allowed to evaporate to dryness, leaving a black tarry residue with a layer of liquid fatty material on the surface. The mass was washed thoroughly with several portions of petroleum ether to leave a dark green powder, which was further washed with ethyl ether until the washings were practically colorless. The residue was dissolved in 50 cc. of methanol, 50 cc. of chloroform was added, and the mixture shaken with 250 cc. of water. The chloroform was drawn off and the aqueous portion washed three times with 50-cc. portions of chloroform. The chloroform extracts were evaporated to dryness, the residue dissolved in methanol, methyl isobutyl ketone added, and the mixture allowed to crystallize. The material which separated was filtered off and washed with methyl isobutyl ketone until the washings were colorless. The dried material, which melted at 185–187°, was combined with the material previously separated. The mother liquor and washings were combined, allowed to evaporate to dryness, and the residue largely decolorized by dissolving in methanol and refluxing for fifteen minutes with charcoal. The filtered solution was concentrated and, after the addition of methyl isobutyl ketone, allowed to crystallize. The material which separated was different from that previously obtained. In addition to its greater solubility, it showed a different melting-point picture and crystal structure. This material will be referred to as the low-melting material.

Purification of High-Melting Material.—The crystalline material melting above 180° was washed three times with petroleum ether to remove a white waxy material. The washed glycosides were further purified in the following manner: the glycosides were dissolved in methanol in a ratio of 1 Gm. in 25 cc. The solution was filtered and diluted with water to reduce the alcohol concentration to 16%; then extracted four times with methyl isobutyl ketone using an amount equal to one-fourth the volume of methanol-water for each extraction. Both the ketone extract and the aqueous portion were concentrated to a small volume under reduced pressure with a bath temperature of not over 60°. The material which crystallized from the ketone as well as the residue left on allowing evaporation to dryness was white or light tan in color. The aqueous concentrate also deposited some crystalline material, white in color, with a melting point and a Keller reaction identical to that of the material from the ketone extracts. It was combined with the ketone material. The aqueous concentrate, on being allowed to evaporate to dryness, left a syrupy green residue which showed a red-brown ring with the Keller reagent, but only a faint blue-green color in the upper layer. Attempts to obtain crystalline material from this residue yielded only sticky brown syrups. A sample of the glycosides crystallized twice from aqueous methanol and three times from methanol-methyl isobutyl ketone had a melting point of 198–200°. Since the melting point depended greatly upon the method of heating, a standard procedure of preheating the bath to 180° before introduction of the sample at the rate of 6° per minute was adopted. All the high-melting material was crystallized from methanol-methyl isobutyl ketone until a white product melting at 198–200° was obtained. It was necessary to decolorize several of the later crops with charcoal in order to obtain a white product. The Keller reaction of the material now showed a brown ring with a tinge of red at the lower face and the typical blue of digitoxose in the acetic acid layer.

In their investigation of the digilanids, Stoll and Kreis (3) were able to effect a separation of the individual glycosides by partitioning between chloroform and aqueous methanol. When their procedure was applied to our material, little or no separation of the gitoxigenin-containing material, indicated by the red tinge given to the Keller reaction, was achieved. Three successive partitionings gave partition coefficients for the chloroform fraction of 97.7, 98.0, and 97.9. There was no detectable difference in the color reactions of the successive fractions or between the second and third chloroform fractions and the third water-methanol fraction. The partitioned material was crystallized several times from methanol. The melting point remained at 198–200°.

Since the residues of the methanol mother liquors showed the presence of the low-melting material previously noted, the high-melting material was subjected to the following procedure to completely free it of this material. The low-melting component was found to be somewhat soluble in ether, therefore the high-melting material was extracted for six hours in a Soxhlet apparatus with this solvent. A weight loss of 6.7% was noted. Evaporation of the ether extract revealed that the residue consisted of low-melting material. Further separation was effected by crystallizing the high-melting material several times from a mixture of equal volumes of chloroform and methanol, to which ether was added to the

point of crystallization. The mixture was allowed to stand in the refrigerator overnight before removal of the crystals. The low-melting material does not crystallize from this mixture.

An attempt to determine quantitively the amount of gitoxigenin-containing material present by the method of McChesney, *et al.* (5), indicated the presence of material different from gitoxigenin and digitoxigenin. Figure 1 illustrates the spectra in concentrated sulfuric acid of the genins prepared from 20-mg. samples of high-melting material and of digitoxin and gitoxin.[1] In addition, a 25.3-mg. sample of high-melting material was used in order to get approximately molar comparison based on the molecular weight of digilanid A. The differences suggested the possible presence of digoxigenin, but unfortunately no digoxigenin-containing materials were available for comparison. The lack of a standard made impossible a quantitative estimation of the gitoxigenin present. These and all other colorimetric readings were taken with a Coleman Model 6A electrophotometer using round 19-mm. Coleman Series B cells matched to within 0.3% transmission with distilled water.

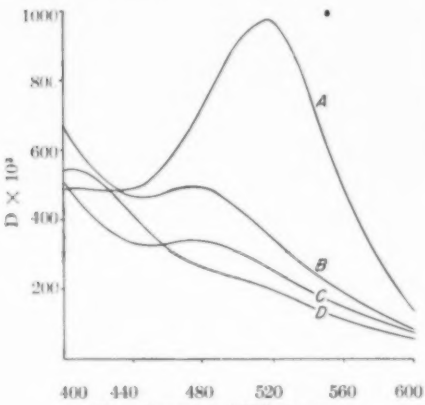

Fig. 1.—Genins in concentrated sulfuric acid. *A*, gitoxigenin; *B*, unknown (from 25.3 mg.); *C*, unknown; *D*, digitoxigenin.

Characteristics of the High-Melting Material (Acetyl Digoxin).—The typical melting point in a bath preheated to 180°, as mentioned above, showed some softening at 192° and a sudden decomposition at 197–198° (203–204°, corr.). The melting point was not changed after four hours of drying at 110° in a vacuum over phosphorus pentoxide. The material crystallized from chloroform-methanol-ether in the form of rectangular platelets (Fig. 2). From methanol or aqueous methanol, the crystals were long, needle-like six-sided prisms with a slanted tip (Fig. 3). The optical rotation of samples dried at 76° in vacuum and dissolved in 95% ethanol was +25 ± 2°. No nitrogen, sulfur, or halogens were

[1] Samples of digitoxin and gitoxin specially purified for research use were obtained through the courtesy of Dr. E. W. McChesney, Sterling-Winthrop Research Institute, Rensselaer, N. Y. These were used as references throughout this study.

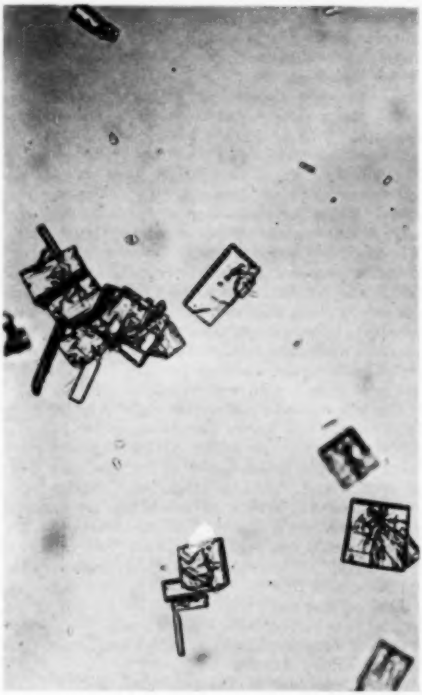

Figure 2

present. The Baljet and Legal tests were positive. The ultraviolet absorption was not different from that of digitoxin or gitoxin. In all cases the peak of absorption lay beyond the range of the instrument.

With a view toward getting an insight into the composition and molecular size, the material was subjected to a quantitative Keller-Kiliani determination to measure the digitoxose present (Fig. 4). The procedure followed was that of the U. S. P. XIII (6) method II for the determination of digitoxin. The sample size and quantities of reagents were doubled in order to achieve the same concentrations used in the outlined method and have a volume large enough to fill the cells used. Digitoxin and digilanid A[2] were used as reference standards. The results based on the digitoxose content of digitoxin showed 45.9% of digitoxose in the unknown, calculated for acetyl digoxin 47.4%. For digilanid A, the value found was 24.9%, the calculated value for two digitoxose groups being 26.8% [only two of the digitoxose groups of digilanid A give a color reaction; the third, which is bound to a glucose residue does not respond (3)]. By application of this procedure to the genins, their influence on the color obtained was found to be insignificant. The molecular weight of the high-melting material calculated from the curve obtained and using digitoxin as a

[2] The reference sample of digilanid A was obtained through the courtesy of Dr. A. Stoll, University of Basel, Switzerland.

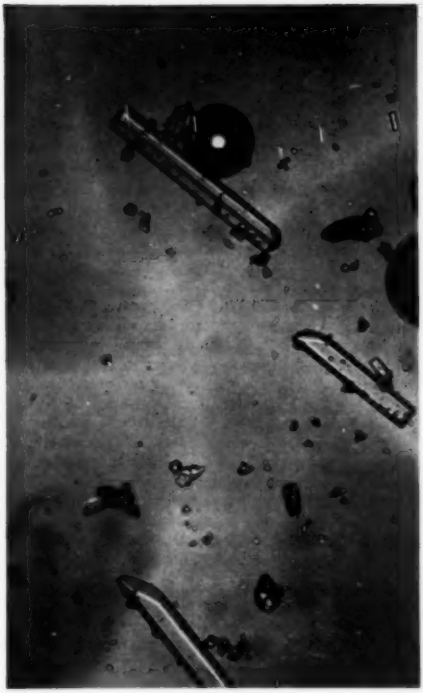

Figure 3

basis for calculation was 828; calculated for acetyl digoxin, 823.

A quantitative Baljet determination, U. S. P. XIII method I (6) gave values for the high-melting material which fell below those of digitoxin and of digilanid A, indicating that either the molecule was larger than digilanid A or that the genin was different from digitoxigenin (Fig. 5). It has been shown (7, 8) that glycosides containing gitoxigenin or digoxigenin fail to give as intense a color reaction as the digitoxigenin-containing compounds. Because of the lack of a reference standard containing digoxigenin, no quantitative conclusions could be drawn.

Anal.—Calcd. for acetyl digoxin, $C_{43}H_{66}O_{15}$: C, 62.75; H, 8.08. Found[2]: C, 61.85, 61.95, 62.01; H, 8.17, 8.35, 7.94.

De-esterified Product (Digoxin).—A lactone titration of 0.2-Gm. samples with 0.02 *N* sodium hydroxide by the method of Windaus and Schwarte (9) gave values of 521 and 528, indicating the presence of two alkali-consuming groups, one of which would be the lactone group of the genin. Stoll and Kreis (10) have shown that it is possible to deacetylate the digitalis glycosides without opening the lactone ring by using dilute alkali in cold methanol solution. By this procedure, a 0.2-Gm. sample of glycoside in ice-cold methanol was de-esterified with 0.1 *N* sodium hydroxide. The resulting product was

[2] We are indebted to Dr. A. E. Osterberg, Abbott Laboratories, North Chicago, Ill., for some of the carbon-hydrogen analyses.

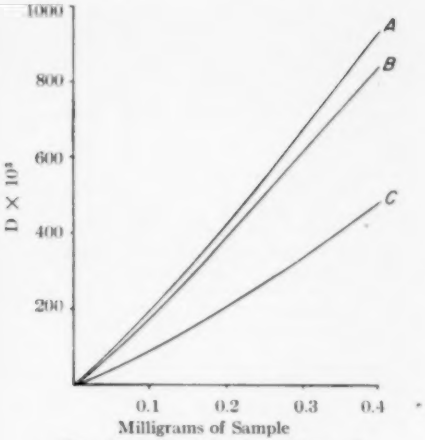

Fig. 4.—Keller-Kiliani reaction. *A*, digitoxin; *B*, unknown; *C*, digilanid A.

a white microcrystalline powder. The Baljet and Legal tests were positive, indicating an intact lactone ring. The Keller reaction gave a brown ring with a faint trace of red, the acetic acid layer becoming blue. The melting point in a bath preheated to 220° was 253–255° (262–264°, corr.); reported for digoxin, 265° (corr.) (11). The rotation of a 0.0373-Gm. sample in 5 cc. of 75% ethanol (c = 0.756) observed in a 1-dm. tube was $+0.100 \pm 0.011$, $[\alpha]_D^{25} = +13.2 \pm 1.5°$; reported for digoxin, +13.3 (11). A quantitative Keller-Kiliani determination showed 49.9% of digitoxose; calculated for digoxin, 50.0%. The molecular weight calculated from the curve obtained was 786; calculated for digoxin, 781.

Anal.—Calcd. for digoxin, $C_{41}H_{64}O_{14}$: C, 63.18; H, 8.26. Found: C, 62.99; H, 8.54.

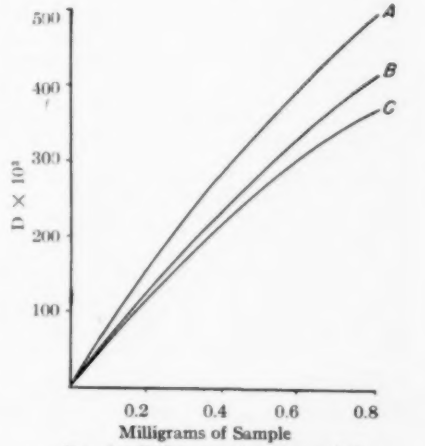

Fig. 5.—Baljet reaction, tetraethylammonium hydroxide as base. *A*, digitoxin; *B*, digilanid A; *C*, unknown.

Hydrolysis of High-Melting Material.—A 0.9065-Gm. sample was hydrolyzed by refluxing in a mixture of 90 cc. of ethanol and 90 cc. of 0.1 *N* sulfuric acid for thirty minutes on a steam bath. The cooled solution was neutralized with washed barium carbonate, filtered, and the residue washed with two 25-cc. portions of ethanol followed by two 25-cc. portions of water. The filtrate and washings were concentrated at reduced pressure and a temperature of 40° to a volume of 100 cc. The concentrate was extracted with three 50-cc. portions of chloroform which were combined and washed twice with 50-cc. portions of water to remove any sugars which may have carried over into the chloroform. The chloroform extract was concentrated at reduced pressure and a temperature of 40° to a small volume; then transferred to a tared evaporating dish and dried in a stream of air. The residue, which was of a syrupy nature, weighed 0.2247 Gm. after drying for an hour at 90°. The Keller test showed a yellow-brown ring with a strong carmine-red color in the acetic acid. Attempts made to crystallize the residue from aqueous ethanol resulted only in a gum. When crystallized from ethyl acetate, 0.0525 Gm. of crystalline genin was obtained. A Keller test on the crystals showed a yellow-brown ring with a strong red line at the bottom. The ethyl acetate mother liquors left a gummy residue when allowed to evaporate to dryness.

Further concentration of the hydrolysis mixture caused the separation of much flocculent material which was removed by filtration through a tared Gooch crucible, washed well with water, and dried for an hour at 90°. The residue weighed 0.2843 Gm. It was extracted with five 30-cc. portions of boiling chloroform from which needles separated on cooling. The chloroform extract was allowed to evaporate to dryness leaving a crystalline residue which weighed 0.2725 Gm. This material gave a yellow-brown ring with a trace of red when tested with the Keller reagent. There was no trace of a blue color. The material was difficultly soluble in chloroform, from which it crystallized as ragged needles. When melted, they effervesced at 135–140°, then solidified and melted at 203–205°. Three crystallizations from aqueous ethanol resulted in the same irregular needles with the same melting-point behavior. When dried at 110° for four hours in a vacuum, the melting point was 203–205°. Mannich and Schneider (12) have reported a similar behavior for digoxigenin. The genin was recrystallized twice from ethyl acetate, resulting in thick prisms which with the Keller reagent showed a clear yellow-brown ring with no trace of red, indicating the removal of the possible gitoxigenin. The air-dried genin melted at 206–207°. When vacuum dried at 110°, the melting point was 210–214° (216–220°, corr.); reported for digoxigenin 222° (corr.) (11). The rotation in a 1-dm. tube of a 0.0527-Gm. sample dissolved in 5 cc. of methanol ($c = 1.054$) was $+0.253 \pm 0.018$, $[\alpha]_D^{25} = +24.0 \pm 2.0°$; reported for digoxigenin, +25.8° (11), +25.3°, +24.6° (12).

Anal.—Calcd. for digoxigenin, $C_{23}H_{34}O_5$: C, 70.74; H, 8.78. Found: C, 70.70, 71.02; H, 8.86, 8.65.

The remaining hydrolysis mixture was concentrated to a yellow syrup which was dried by adding and evaporating off several portions of absolute ethanol. It was then dried in an evacuated desiccator over Drierite for forty hours and extracted with absolute ethanol, leaving a small amount of inorganic residue. After removal of the ethanol, the syrup was dried for three days in an evacuated desiccator. It weighed 0.4754 Gm. To insure complete removal of the ester grouping, the syrup was heated at 50° for three hours with 2 cc. of 0.1 *N* sodium hydroxide. The mixture was acidified with 0.1 *N* sulfuric acid and the excess acid neutralized with washed barium carbonate. The mixture was filtered, concentrated to a syrup, dried, and extracted with absolute ethanol as before. All of the syrup was soluble in absolute ethanol. It gave a strong digitoxose reaction with the Keller reagent. A Molisch test gave no indications of the presence of glucose. Although the sugar could not be brought to crystallization, its benzimidazole derivative was prepared and found to correspond to that reported for digitoxose. The method used was that of Dimler and Link (13), and following this procedure, only a small amount of material was obtained. Therefore, the reaction mixture was evaporated to dryness and extracted with several portions of hot ethyl acetate from which the benzimidazole separated as dirty brown crystals which melted at 201–203°. They were decolorized with charcoal and recrystallized from methanol, resulting in platelets which melted at 208–210°. Reported for digitoxose benzimidazole, 207–209° (13).

Anal.—Calcd. for $C_{12}H_{16}N_2O_3$: C, 61.00; H, 6.83. Found: C, 60.69; H, 6.71.

The absolute ethanol-insoluble residues of the sugar concentrate and the mother liquors from the de-esterification procedures were combined, acidified with 0.1 *N* sulfuric acid, and steam-distilled. The distillate was neutralized with 0.1 *N* sodium hydroxide, evaporated to dryness, and the phenylphenacyl ester of the acid was prepared. The ester was crystallized from aqueous ethanol. Two crops of crystals which gave a positive Beilstein test were discarded. Removal of most of the ethanol by heating resulted in crystallization of the ester. It was separated and crystallized several times from heptane as platelets which melted at 108.5 to 109.5° and gave a negative Beilstein test. The only volatile acid forming a phenylphenacyl ester which melts near this point is acetic acid, the ester of which melts at 110–111° (14).

Characteristics of the Low-Melting Material (β-Acetyl Digoxin).—The residues of the ketone mother liquors from which the high-melting material had been crystallized were dissolved in methanol and decolorized with charcoal. The solution was filtered, evaporated to dryness, and the residue dissolved in a mixture of equal volumes of methanol and chloroform. Ether was then added to precipitate the high-melting material. After a day in the refrigerator, the mixture was filtered and the filtrate evaporated to dryness. The residue was refluxed with ether, the solution filtered and the ether distilled off. The residue was crystallized several times from aqueous methanol. By slow crystallization, rosettes of long silky needles could be obtained. These resembled the crystals pictured by Stoll (15) for β-acetyl digoxin. The melting point did not change on recrystallization or on vacuum drying. The material began softening at 156° until it became a thick jell at 175°; no further change occurred until the jell

began to decompose and liquefy at 200°; liquefaction was complete at 220° (160–227°, corr.), though decomposition continued to 240°. Reported for β-acetyl digoxin, 170–258° (corr.) (16). The partition coefficient between chloroform and aqueous methanol determined in the same manner as for the high-melting material was 96.5. The Keller test gave a brown ring with a tinge of red accompanied by the characteristic blue of digitoxose in the acetic acid layer. The Baljet and Legal tests were positive. No nitrogen, sulfur, or halogens were indicated. The optical rotations were as follows: 0.2243 Gm. in 10 cc. of chloroform (c = 2.243) observed in a 1-dm. tube rotated +0.633 ± 0.086, $[\alpha]_D^{20}$ = +28.2 ± 4.0°; 0.2240 Gm. in 25 cc. of 95% ethanol (c = 0.896) observed in a 2-dm. tube rotated +0.549 ± 0.030, $[\alpha]_D^{20}$ = +30.6 ± 2.0°. Reported for β-acetyl digoxin, +29.2° (16), +30.4° (12). A quantitative Keller-Kiliani determination showed 49.0% of digitoxose; calculated for acetyl digoxin, 47.4%. The molecular weight calculated from the curve obtained was 802; calculated for acetyl digoxin, 823. A Baljet determination gave a curve almost identical to that of the high-melting material. The small size of the available sample (0.4 Gm.) prevented a hydrolysis and separation of the genin. Although no attempt was made to determine the presence of an ester grouping by saponification, a 0.2-Gm. sample was subjected to a de-esterification on the assumption that one was present. The product consisted of microcrystalline platelets resembling the product prepared from the high-melting material. The Baljet and Legal tests were positive. The melting point in a bath preheated to 220° was 250–254° (259–263°, corr.). A mixed melting point with the de-esterified product of the high-melting material showed no depression. The rotation of a 0.0332-Gm. sample in 5 cc. of 75% ethanol (c = 0.608) observed in a 1-dm. tube was +0.101 ± 0.017, $[\alpha]_D^{20}$ = +16.3 ± 2.8°.

Anal.—Calcd. for acetyl digoxin, $C_{43}H_{66}O_{18}$: C, 62.75; H, 8.09. Found for low-melting material: C, 61.77; H, 8.19.

Calcd. for digoxin, $C_{41}H_{64}O_{14}$: C, 63.18; H, 8.26. Found for de-esterified product: C, 63.36; H, 8.47.

DISCUSSION

There can be little doubt that the principal genin of the high-melting material, which composed the larger fraction of the glycosides, was digoxigenin. The presence of gitoxigenin was indicated by the red tinge given to the ring in the Keller test, not only by the glycoside but also by the mixture of genins obtained on hydrolysis. No indications of the presence of digitoxigenin in any considerable amount were noted. The presence of acetic acid and the absence of sugars other than digitoxose, together with the formation of digoxin on de-esterification, point to an acetyl digoxin as the high-melting material. When crystallized from methanol-chloroform-ether, the material displays a crystal structure like that pictured by Stoll (15) for α-acetyl digoxin. However, the melting point (197–204°, corr.) lies considerably below that reported for α-acetyl digoxin (220–230°, corr.) (16). This might be attributed to the presence of a gitoxigenin-containing material (reported melting point of acetyl gitoxin, 210–225°, corr.) (16). The observed rotation (+25 ± 2.0°) cannot be explained on this basis since it lies above the reported rotations of both α-acetyl digoxin (+18.0) and acetyl gitoxin (+15.7°) (16). The presence of the low-melting material (melting point, 160–227°; rotation, +29.4) would seem to offer an explanation, yet the large proportion necessary to produce the increase in rotation coupled with the purification expressly intended to remove it, would rule out such a possibility. We are unable to offer an explanation for these discrepancies.

Although the low-melting material showed many indications of its identity with β-acetyl digoxin, the melting point failed to agree exactly with that reported in the literature. The small size of the available sample prevented an extensive examination of the material so no positive identification could be made. A small amount of gitoxigenin-containing material was apparently present, as indicated by the tinge of red imparted to the Keller test.

SUMMARY

1. By a modification of the Bay and Gisvold procedure, crystalline glycosides have been isolated from the fresh leaves of *Digitalis lanata* and separated into two fractions.

2. The higher melting fraction was hydrolyzed and digoxigenin, digitoxose, and acetic acid identified in the hydrolysis mixture. Color reactions also indicated the presence of gitoxigenin.

3. The higher melting fraction was de-esterified, resulting in a product identified as digoxin.

4. The preponderance of evidence indicates that the principal component of the higher melting fraction is an acetyl digoxin. A small amount of a gitoxigenin-containing component also appears to be present.

5. The second, lower melting fraction has been tentatively identified as β-acetyl digoxin admixed with gitoxigenin-containing material.

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Digitalis Extraction Studies. IV*,†

By G. G. KRISHNAMURTY and OLE GISVOLD‡

A crystalline glycosidic substance was isolated, using enzyme-inhibiting techniques, from *Digitalis lanata* that was grown from seeds obtained from A. Stoll. This glycosidic material had physical and chemical properties that very closely resembled those of a glycosidic material obtained from *Digitalis lanata* that had been propagated over a number of years in the botanical gardens of the University of Minnesota. This glycosidic material was previously characterized as being composed chiefly of acetyl digoxin. When fresh leaves were incubated for eight, twenty-four, and forty-eight hours, either the same crystalline glycosidic material was obtained as previously described or no crystalline material could be isolated. *Digitalis lanata* leaves that had been improperly stored for seven months failed to yield any crystalline glycosidic material. By using Stoll's enzyme-inhibiting techniques, a crystalline glycosidic material was obtained that appeared to be identical to that obtained by the new techniques described. In addition, the Stoll technique was more tedious and difficult.

In preceding studies it has been shown that fast-frozen and powdered leaves of *Digitalis lanata* can be extracted by new techniques to yield α- and β-acetyl digoxin as the chief crystalline glycosides (1) when this plant is grown in the Medicinal Plant Garden of the College of Pharmacy of the University of Minnesota. It appeared that the procedure employed prevented any enzymatic activity and that the native glycosides were obtained. These studies have been extended and confirmed with other new data. Stoll (2), in his extensive investigations on the nature of the glycosides found in *Digitalis lanata*, reported the presence of the lanatosides-A, -B, and -C. No mention is made by Stoll or others of the isolation of the acetyl glycosides such as acetyl digitoxin, acetyl digoxin, and acetyl gitoxin from *Digitalis lanata*. These compounds were reported, however, as a result of the *in vitro* enzymatic degradation of the native glycosides. Subsequent deacetylation of these glycosides gave the well-known digitoxin, digoxin, and gitoxin.

Prior to Stoll's studies, Smith (3) had isolated digoxin from *Digitalis lanata* and this glycoside is now available commercially. Although Stoll did not enzymatically remove the acetyl group, one is led to believe that if enzyme-inhibiting techniques are not employed one would obtain, for example, digoxin and not lanatoside-C. Therefore, the loss of the acetyl group as well as the glucose residue can be attributed to enzymatic activity. In the present studies it was of interest to determine if enzymatic activity could remove the acetyl group with the resultant production of digoxin. These studies are not entirely complete and it is hoped that they can be extended. These studies were carried out on both fresh and dried leaves.

Because the *Digitalis lanata* used in this and previous studies had been propagated over a number of years at Minnesota and yielded the apparent acetyl-glycosides as "native" glycosides, it was of interest to obtain seeds of *Digitalis lanata* from Switzerland. Dr. Stoll kindly supplied some seeds of *Digitalis lanata* which germinated well and the plants were grown in the Medicinal Plant Garden. These plants were harvested in the late summer and fall and when investigated by the previously described enzymatic-inhibiting techniques, they yielded crystalline glycosides indistinguishable from those previously reported. Furthermore, the application of Stoll's enzyme-inhibiting techniques, although much more cumbersome and tedious, yielded crystalline glycosides having identical physical properties. It would appear, therefore, that soil and climatic conditions play an important role in determining the nature of the glycoside produced by the plant. This is not an unusual phenomenon; however, to our belief it has been sadly neglected in the case of such important drugs as the *Digitalis* species.

EXPERIMENTAL

Isolation of Glycosides after Enzymatic Action.—No attempt was made to purify or concentrate any enzymes that might be present in the fresh leaves, as it is well known that enzymatic activity can take place in crude preparations, disintegrated cells and, of course, in the intact cell. This has been shown to be true in conjunction with a number of cardiac glycoside studies (4). It was hoped that enzymatic activity could be obtained in the disintegrated leaves. The fresh leaves of *Digitalis lanata* were frozen and

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powdered as previously described (1). The frozen powder was allowed to thaw at room temperature, suspended in distilled water, heated to 40°, layered below with chloroform and above with toluene, and then incubated in an electric oven at 40°. After a given period of time the incubated mixture was heated to 65–70° (not higher) as a most suitable temperature, the mixture filtered, the marc washed with water, and the filtrate extracted with methyl isobutyl ketone as previously described (1). Several trial runs on 250-Gm. batches of fresh leaves were needed to obtain the best conditions in order to minimize the amounts of chlorophyll and other pigments in the methyl isobutyl ketone extracts. In the subsequent experiments 1,000-Gm. batches of fresh frozen leaves were used. When incubation was allowed to proceed for eight hours, 0.75 Gm. of crude crystalline material was obtained as compared to an 0.8-Gm. control with no incubation. Therefore, it appeared that little if any enzymatic activity had taken place. If incubation was allowed to proceed for forty-eight hours at 40°, no crystalline material could be isolated. In addition, the methyl isobutyl ketone extracts were very strongly colored due to chlorophyll, etc., which is not encountered when fresh leaves are worked up immediately. If the incubation period is twenty-four hours, about 0.3 Gm. of a strongly colored semicrystalline material was obtained as a first crop and no subsequent crops were obtained from the concentrated mother liquors. Larger batches were subjected to twenty-four-hour incubation periods in order to obtain enough material to purify for characterization purposes. This was done because it represents a point between which little change has taken place (eight hours) and the time (forty-eight hours) at which no crystalline product could be obtained. Sufficient 1,000-Gm. batches were investigated in order to obtain workable quantities of crude crystalline glycosidic material. This crude crystalline material was continuously extracted successively with petroleum ether and ether in a Soxhlet extractor in order to remove any extraneous material that was soluble in these solvents. The residual extracted glycosidic material was almost free of color and weighed 1.7 Gm. It was partitioned between chloroform and aqueous methanol for purification purposes and to obtain its partition coefficient $Chl./(Chl. + W)$ where *Chl.* represents the weight of the residue from the chloroform layer and *W* the weight of the residue from aqueous-methanol. Repeating this procedure five times, the partition coefficient increased from 93 to 97.9. All fractions when tested by the Keller-Kiliani (5) reaction gave a distinct brown, containing a trace of red, at the interphase. All the chloroform residues melted between 200–205° when placed in a bath preheated to 180°. The fifth fraction that had a partition coefficient of 97.9 was repeatedly recrystallized from a mixture of methanol, chloroform, and ether. No change in the melting point of 200–205° was obtained nor a change in the Keller-Kiliani color test. A positive Baljet (6) and Legal (7) test was obtained. The rotation of a 0.3618-Gm. sample in 50 cc. of alcohol observed in a 2-dm. tube was $+0.4 \pm 0.01$ $[\alpha]_D^{29} = +27.1 \pm 0.68$.

Anal.—Calcd. for acetyl digoxin $C_{43}H_{66}O_{15}$: C, 62.75; H, 8.08. Found: C, 62.32; H, 7.79.

Further purification by partitioning and crystallization followed by analyses failed to alter the above values significantly.

It can be reasonably concluded that the above crystalline glycoside is very similar to that isolated from the fresh leaves employing enzyme-inhibiting techniques. The mother liquors from which the crystalline glycoside was obtained did not appear to contain the possibly expected digoxin.

Examination of Leaves of Digitalis lanata About One Year Old.—2,000 Grams of dried leaves of *Digitalis lanata* that had been stored for about one year under somewhat damp conditions were carefully dried and powdered. These leaves were extracted with hot water (65–70°) to make a 2% extract. This extract had much chlorophyll. It was extracted with methyl isobutyl ketone and the ketone concentrated and manipulated as described when used in conjunction with the extract from fresh leaves. A strongly colored, sticky mass was obtained from which no crystalline material could be obtained. These results were similar to the forty-eight-hour incubation at 40° of the fresh leaves. These results are similar to those observed by others in conjunction with improperly stored leaves.

Examinations of Rapidly Dried Leaves of Digitalis lanata.—Cleaned *Digitalis lanata* leaves were dried at 40° in an electric oven equipped with a blower. They were then powdered in a Jacobson mill to about a No. 80 powder. The dried powder was added to hot water (65–70°) and filtered immediately after the temperature had reached 70°. The marc was repeatedly extracted with hot water (65–70°) to make a 2% aqueous extract. While still warm the aqueous extract was extracted with methyl isobutyl ketone and the ketone extract worked up as previously described (1). The crude crystalline glycoside was exhaustively extracted with petroleum ether and then with ether. The white residue was then purified as described above.

Partitioning between chloroform and aqueous methanol four successive times gave values that increased from 92 to 97.9. All fractions when tested by the Keller-Kiliani (5) reaction gave a distinct brown, containing a trace of red, at the interphase. The fourth fraction that had a partition coefficient of 97.9 when crystallized from a mixture of methanol, chloroform, and ether yielded square platelets and rods with sharp square corners (Fig. 1). When the melting point was taken simultaneously with the product isolated after enzymatic action, both began to shrink at 190° and melted at 200–205°. The rotation of a 0.2270-Gm. sample in 25 cc. of alcohol observed in a 2-dm. tube was 0.48 ± 0.03, $[\alpha]_D^{26} = +26.4 \pm 1.6$.

Anal.—Calcd. for acetyl digoxin $C_{43}H_{66}O_{15}$: C, 62.75; H, 8.08. Found: C, 61.98, 61.87; H, 8.27, 8.40.

It would appear that this compound is very similar, if not identical, to that obtained from fresh leaves.

Isolation of Glycosides from Fresh Leaves of Digitalis lanata.—Powdered fresh fast-frozen leaves of *Digitalis lanata* were examined for the nature of the glycosidic material that could be isolated using the enzyme-inhibiting techniques of Stoll and Kreis (2). Partitioning between chloroform and aqueous

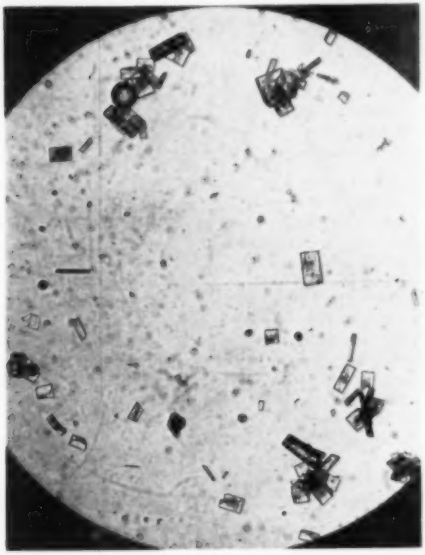

Fig. 1.—Crystals of acetyl digoxin from rapidly dried leaves of *Digitalis lanata*.

Fig. 2—Crystals of acetyl digoxin from fresh leaves of *Digitalis lanata*

methanol four successive times gave values that increased from 95.9 to 98. No intermediate phase was obtained (lanatoside-B) and no aqueous methanol soluble fraction was obtained (lanatoside-C). All fractions gave a positive Keller-Kiliani (5) reaction in which only a trace of red was admixed with the brown interphase. The fourth fraction that had a partition coefficient of 98 was recrystallized from a mixture of methanol, chloroform, and ether to give rectangular platelets and rods (Fig. 2). The crystalline glycoside melted at 200–205°. The rotation of a 0.1033-Gm. sample in 10 cc. of alcohol observed in a 1-dm. tube was $0.28 \pm 0.01°$, $[\alpha]_D^{25} = +27.1 \pm 1$.

Anal.—Calcd. for acetyl digoxin, $C_{43}H_{66}O_{15}$: C, 62.75; H, 8.08. Found: C, 61.60; H, 8.02.

It would appear that this glycoside or material is very similar, if not identical, to that obtained from the fresh leaves by the new techniques developed.

Isolation of the Glycosides from Minnesota-Grown Digitalis lanata (Swiss Seed).—These plants appeared to be identical to the plants obtained from those of *Digitalis lanata* that had been previously propagated from year to year in the Medicinal Plant Garden of the College of Pharmacy, University of Minnesota. They were freshly collected and examined phytochemically as previously described here and elsewhere (1). The recrystallized product (Fig. 2) had a partition coefficient of 98 and melted at 200–205°. The rotation of a 0.2253-Gm. sample in 25 cc. of alcohol in a 2-dm. tube was $+0.46 \pm 0.02$, $[\alpha]_D^{25} = +25.5 \pm 1.1$.

Anal.—Calcd. for acetyl digoxin $C_{43}H_{66}O_{15}$: C, 62.75; H, 8.08. Found: C, 62.19; H, 8.19.

The physical properties and analyses of this crystalline glycosidic product compared favorably with those of the products isolated from variously treated samples of Minnesota-grown *Digitalis lanata*.

SUMMARY

1. Fresh, disintegrated leaves of *Digitalis lanata* were incubated at 40° for eight, twenty-four, and forty-eight hours. After eight hours only a small decrease in the amount of isolatable crystalline glycosidic material was obtained. No crystalline glycosidic material could be isolated after forty-eight hours, whereas after twenty-four hours some crystalline glycosidic material could be isolated. This crystalline material appeared to be identical to that obtained when fresh leaves and enzyme-inhibiting techniques were employed.

2. No crystalline glycosidic materials could be isolated from leaves of *Digitalis lanata* that had been improperly stored for seven months.

3. Powdered leaves of *Digitalis lanata* that had been carefully and rapidly dried yielded a crystalline glycosidic material identical to that obtained from the fresh leaves.

4. The crystalline glycosidic material obtained from fresh *Digitalis lanata* leaves using A. Stoll's enzyme-inhibiting and isolation techniques appeared to be identical to the crystalline glycosidic material previously isolated from fresh

Digitalis lanata leaves by much simpler techniques.

5. Fresh *Digitalis lanata* leaves obtained from plants grown from seed obtained from Switzerland (through the courtesy of A. Stoll), when examined by enzyme-inhibiting techniques, yielded a crystalline glycosidic material that appeared to be identical to that previously isolated from fresh *Digitalis lanata* leaves.

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Some Derivatives of *o*-Thymotinic Acid*,†

By SHAO-CHIA CHOU, PAUL J. JANNKE, JOSEPH B. BURT, and LaVERNE D. SMALL

An improved method for the preparation of *o*-thymotinic acid is given. The preparation and results of analysis of some new derivatives of the acid are included.

THE structural relationship of *o*-thymotinic acid to thymol and to salicylic acid was noted by Street, Georgi, and Jannke (1). Since both thymol and salicylic acid had long been known to possess antiseptic properties, these workers prepared a number of salts of *o*-thymotinic acid and tested their bactericidal and fungicidal action. The results of this investigation prompted further study of this acid.

o-Thymotinic acid was first prepared by Kolbe and Lautemann (2) in 1860, by heating thymol, metallic sodium, and carbon dioxide in a closed container at 230°. This paper reports an improved method for the preparation of *o*-thymotinic acid giving slightly higher yields and a purer product than previously reported (1).

A number of derivatives of *o*-thymotinic acid have now been prepared by substituting various groups for hydrogen attached to the aromatic nucleus of the acid.

Because of recent interest in the use of *p*-aminosalicylic acid for the treatment of tuberculosis (3), the aminothymotinic acid hydrochloride was prepared. The aminothymotinic acid proved to be unstable; hence, the stable amine hydrochloride was prepared for testing against tuberculosis. The results of this investigation as well as studies on the bactericidal and fungicidal activity of these compounds will be reported later.

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EXPERIMENTAL

***o*-Thymotinic Acid.**—The *o*-thymotinic acid was prepared by a modification of the method reported by Street, Georgi, and Jannke (1), wherein granulated sodium is added to a solution of thymol in boiling xylene under an atmosphere of dry carbon dioxide. The equipment used was essentially the same as that reported by these workers, except that the reaction was heated by means of an electric mantle.

Six hundred grams (4 moles) of thymol was dissolved in 7.5 L. of dry xylene and the solution was heated to the boiling point. Granulated sodium was added in small amounts while the boiling solution was stirred, and a continual stream of dry carbon dioxide gas was bubbled into the reaction mixture. A total of 190 Gm. (8.25 moles) of sodium was added within two hours. Heating was continued at such a rate as to maintain gentle refluxing, and the flow of carbon dioxide was continued for about nine hours more, after which the reaction mixture was allowed to cool to room temperature. A few cubic centimeters of distilled water was added through the top of one of the condensers at intervals of a few minutes, accompanied with stirring and a rapid flow of carbon dioxide. Water was added until all the sodium metal was decomposed and the product was dissolved in the aqueous layer. The aqueous layer was then separated and the *o*-thymotinic acid was precipitated by the addition of hydrochloric acid (sp. gr. 1.09). The white crystalline *o*-thymotinic acid was collected on a suction filter, washed with water and dried; the yield was 72% of theoretical. After recrystallization from hot 50% acetic acid, filtering through Norit, and a second recrystallization from petroleum ether (b. p. 60–70°), fine white needle-like crystals melting at 126.5° were obtained. The melting point reported by Sondern was 127° (4).

Sulfothymotinic Acid.—Twenty grams (0.103 mole) of *o*-thymotinic acid was dissolved in 100 cc. of dry chloroform and the solution cooled in an ice bath. Eight cubic centimeters (0.12 mole) of chlorosulfonic acid was added dropwise, with stirring, to the chloroform solution. After standing at room temperature for twenty minutes, the yellow liquid

solidified to a white paste. This paste was pressed on a suction filter and dissolved in a minimum amount of water at 25°. The solution was concentrated under reduced pressure until crystallization started and then placed in a desiccator over concentrated sulfuric acid. The air was partially evacuated from the desiccator and the desiccator was placed in the refrigerator to facilitate further crystallization. Very fine white crystals separated. This crystallization procedure was repeated four times. Care was taken to avoid, as much as possible, exposure of the crystalline product to light. It was found that the sulfothymotinic acid became pink colored when exposed to light for a short time. Weight of pure acid obtained, 10.3 Gm. (37%). It melted at 97–99°. At 100–110° it decomposed and free *o*-thymotinic acid separated.

Anal.—Calcd. for $C_{11}H_{14}O_6S$: S, 11.67. Found: S, 11.60.

Sodium *o*-Thymotinic Acid Sulfonate.—Five grams (0.02 mole) of sulfothymotinic acid was dissolved in 20 cc. of water and a saturated solution containing 2 Gm. (0.03 mole) of sodium chloride was added. After concentration under reduced pressure, white crystals separated. These, following four recrystallizations from absolute alcohol, failed to melt at 300°. The yield was 2.3 Gm. (43%).

Anal.—Calcd. for $C_{11}H_{11}O_6SNa$: Na, 7.77. Found: Na, 7.97.

Sulfothymotinic Acid-*p*-Toluide.—Sodium *o*-thymotinic acid sulfonate, 2 Gm. (0.007 mole) was dissolved in a minimum amount of boiling water. To this solution 1 Gm. (0.01 mole) *p*-toluidine and 4.0 cc. (0.05 mole) hydrochloric acid (sp. gr. 1.19) were added, followed by sufficient hot water to effect solution. The reaction mixture was then allowed to cool, whereupon fine light yellow crystals separated. After recrystallization from 95% ethanol, the compound melted at 225°.

Anal.—Calcd. for $C_{18}H_{23}NO_6S$: S, 8.39; N, 3.67. Found: S, 8.76; N, 3.89.

Amino-*o*-Thymotinic Acid Hydrochloride.—Twenty-five grams (0.105 mole) of nitro-*o*-thymotinic acid, prepared by the method of Sondern (4), was mixed with 300 cc. of hydrochloric acid (sp. gr. 1.19). The mixture was maintained at 60–70° while 101.5 Gm. (0.45 mole) of stannous chloride was added in portions. Several drops of octyl alcohol were added to the reaction to prevent foaming. This reaction was conducted under an atmosphere of carbon dioxide. The temperature was maintained at 60–70° for one and one-half hours after adding the stannous chloride. The mixture was rapidly cooled and 300 cc. of alcohol was added. The amine hydrochloride was collected on a filter. The compound was then recrystallized by dissolving in a small amount of hot water and filtering. The filtrate was collected in a beaker containing 10 cc. of hydrochloric acid (sp. gr. 1.19). The amine hydrochloride separated as fine white crystals weighing 15.3 Gm. (59% of theoretical) and melting at 233° with decomposition.

Anal.—Calcd. for $C_{11}H_{13}NO_3 \cdot HCl$: N, 5.70. Found: N, 5.54.

Iodothymotinic Acid.—The iodo derivative of *o*-thymotinic acid was prepared by the procedure described by Cook and Martin (5) for the preparation of diiodothymol.

Ten grams (0.05 mole) of *o*-thymotinic acid was dissolved in a solution of 4.1 Gm. (0.103 mole) of sodium hydroxide contained in 100 cc. of water. A solution containing 20.0 Gm. (0.16 atom) iodine and 40.0 Gm. (0.24 mole) of potassium iodide in 400 cc. of water was added to the sodium thymotinate solution. A white semisolid precipitate was separated by decantation. After standing overnight, the white semisolid product was warmed to about 50° for ten minutes with hydrochloric acid (sp. gr. 1.19). No immediate change was observed. However, after standing in a desiccator over calcium chloride for several hours, the compound changed to a brown syrup. Crystallization failed to take place after standing in the refrigerator for several weeks. The brown syrup had the following properties: $d_4^{25°}$ 1.661; $n_D^{25°}$ 1.6212. The iodine content, determined gravimetrically as silver iodide, was found to be 39.70%. The iodine content calculated for ($C_{28}H_{34}I_2O_8$) is 39.81%. This formula is comparable to that assigned to thymol iodide, N. F. IX (6).

SUMMARY

An improved method for the preparation of *o*-thymotinic acid, giving a purer product in a shorter time, has been described.

The preparation of five new derivatives of *o*-thymotinic acid has been described and their antibacterial activity is to be studied. The new compounds are:

Sulfothymotinic acid
Sodium thymotinic acid sulfonate
Sulfothymotinic acid-*p*-toluide
Aminothymotinic acid hydrochloride
Iodothymotinic acid.

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A Comparative Study of Tablet Disintegrating Agents*,†

By HERBERT MICHEAL GROSS and CHARLES H. BECKER‡

A comparative study has been made of disintegrating agents commonly used in tablet manufacture. In addition to the accepted agents used by the pharmaceutical industries, some new agents were studied. Tablets were compressed including various disintegrating agents. These tablets were tested for time of disintegration immediately after manufacture and after aging. The results indicate that two new agents, sponge and citrus pulp, compare favorably with those agents commonly used in the tablet manufacturing industry. Data from aging tests indicate loss in disintegrating power by some agents at elevated and normal temperatures, but little effect at reduced temperature.

THE PURPOSE of this investigation was to make a comparative study of disintegrating agents commonly used in tablet manufacture. In addition to the accepted agents used by the pharmaceutical industries, some new agents were found and studied. This investigation was limited to the preparation of tablets by the wet granulation method.

Although a little material has been published on various disintegrating agents, no work of a comparative nature has been reported. Without doubt, manufacturers may have accomplished much of this research, but their findings are so important to their own individual products and markets that they do not publish results.

One of the essential qualities of a good tablet is the ease of its disintegration (10). A tablet that is properly made must readily disintegrate so that the medicament may be utilized. In order that this may be accomplished, it is necessary to add a substance to the tablet formula which will swell or react with the medium into which it is introduced, thus causing the tablet to break apart. This agent is called the "disintegrator." Some coated tablets have disintegrating agents included in their coatings to hasten disintegration (9). Time of disintegration is an essential element in effective medication.

At present, three known methods exist to cause tablets to disintegrate by the use of agents. They are: (*a*) the addition of a gum or carbohydrate material that will swell when moisture is present and thus rupture the tablet; (*b*) the addition of a peroxide that will react with the medium forming oxygen and thus break the tablet; and (*c*) the inclusion of small amounts of acids and carbonates or bicarbonates which when wetted will produce carbon dioxide upon reaction and disintegrate the tablet. Each of these methods in various combinations was included in this investigation.

In an investigation of a comparative nature it is desirable to make constant the variable conditions that may exist for the problem. Such essential conditions as hardness of tablets, speed of compression, nature of lubricant and binder, granulating procedures, and the percentage, nature, and dryness of disintegrating agents must be considered. Standards must be set to account for and control each of these variables.

For comparative purposes, two tablets made by manufacturers were tested and included in compiling the data. The tablets chosen were Bayer's aspirin and Hoffmann-La Roche's Syntrogel. These tablets were selected since they are known to disintegrate quickly.

REVIEW OF THE LITERATURE

A search of the literature revealed that little or no work had been reported on disintegrating agents or on comparative studies of these materials. This can be understood when one realizes that tablet manufacture is a highly recognized art, and information regarding the tablet field has been carefully guarded by the pharmaceutical industry itself. However, the information found in the literature was seriously considered in the research of this problem.

The percentage of disintegrating agent used is dependent upon the action of the agent itself. In general, 5–20% is recommended for starches and gums. Remington (1) suggests the addition of 5% of the agent with the lubricant after the granulation.

The hardness of the tablet is one of the prime factors governing the time of disintegration. A constant compression pressure and set rate of compression will eliminate errors from this source.

The type of lubricant used is important in disintegration time. Berry (2) reports that food products generally cause tablets to disintegrate more rapidly than wetting agents, soaps, or chemicals even though

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the latter may be in a higher percentage in the tablet as a lubricant.

Some factors influencing time rate were noted by Hoyle (3) and Berry (4). They state that the granulation formula, size and weight of tablets, variation in size of granules in granulation, compression, temperature and humidity during manufacture, mechanical machine errors, particle size of medicament, ingredients, and types of hopper-feed vary the disintegration time.

The rapidity of disintegration of tablets composed of two or more ingredients varies directly with the difference in solubility of the ingredients. The reason for this is that the more soluble particles are first attacked by the solvent and form a honeycomb effect thus destroying the cohesion of the less soluble portions and exposing more surface to solution (5).

Schroff (6) suggests the disintegration time rate be checked after a period of time has elapsed from manufacture to account for storage, packaging, aging, and shipping.

EXPERIMENTAL

Materials Used.—The disintegrating agents used are listed in Table I.

The agents of commercial grade listed in Table I were used since at the time of research no better grades were available. In tablets containing calcium carbonate with acids as disintegrating agents, each tablet contained 0.030 Gm. of calcium carbonate with an equimolar quantity of the acid.

Dried citrus pulp and powdered sponge, both natural Florida products, were prepared in the laboratory for this research.

Dried citrus pulp was prepared by washing of the natural waste product from the concentrated juice industry until all sugars and other soluble matter were removed. The pulp was then dried at 40° and ground to a usable fineness. It was then passed through a No. 40 sieve (U. S. Standard).

Powdered sponge was prepared by washing and picking sponge (*Hippospongia lachne*, Delaubenfels) free of foreign material, drying at 40°, grinding to suitable fineness, and passing through a No. 40 sieve.

The binder, a component of the granulating liquid, was zein (corn protein) made by the Corn Products Refining Company. It was of food grade and 5% was dissolved in the granulating liquid.

Lactose U. S. P. was used as the inert bulk of the tablet.

Two per cent leucine was used as the lubricant throughout the investigation.

Two granulating liquids were used. Granulation Formula No. 1 was used with disintegrating agents which would react with water if present. It contained: zein, 5%; isopropyl alcohol (94%), enough to make 100% by volume.

Granulation Formula No. 2 was used with the other disintegrating agents. The inclusion of alcohol aided in drying of the granulation, for upon evaporation alcohol carries with it a greater percentage of water. It contained:

Syrup, 1 part by volume
Water, 1 part by volume
Alcohol, 2 parts by volume
Zein, 5% by weight

Procedure of Manufacture of Tablets.—Two procedures of tablet preparation were followed in the addition of the agents. In the latter procedure some of the disintegrating agent was added with the lubricant after granulation. A control tablet containing no agent was prepared.

Procedure No. 1

(*a*) Materials weighted and sifted separately through a No. 20 sieve (U. S. Standard).
(*b*) Mixed lactose with all the disintegrating agents.
(*c*) Sifted through a No. 16 sieve (U. S. Standard).
(*d*) Granulated with granulating solutions 1 or 2 using a No. 6 sieve (U. S. Standard) for passing the wet granulation through, to establish granule size.

TABLE I.—DISINTEGRATING AGENTS

Agent	Manufacturer	Grade	% Used
Gelloid 50	Jacques Wolf & Co.	Food	17
Pectin	.....	N. F.	17
Methylcellulose, 100 c. p. s.	.....	N. F.	17
Methylcellulose, 4000 c. p. s	.....	N. F.	17
Corn starch	.....	U. S. P.	17
Locust bean gum	S. B. Penick & Co.	Food	17
Karaya gum	.....	N. F.	17
Algin	Algin Corp. of America	Food	17
Sodium alginate	.....	N. F.	17
Veegum HV	R. T. Vanderbilt Co.	Food	17
Bentonite	.....	U. S. P.	17
Aveeno	Quaker Cats Co.	Food	17
C. M. C. 70	Hercules Powder Co.	Food	17
Gum tragacanth	.....	U. S. P.	17
Powdered sponge	.....	Prepared	17
Dried citrus pulp	.....	Prepared	17
Magnesium peroxide	.....	Food	14
Sodium carbonate peroxide	Becco Sales Co.	Commercial	14
Sodium pyrophosphate peroxide	Becco Sales Co.	Commercial	14
Calcium carbonate	.....	U. S. P.	14
with citric acid	.....	U. S. P.	..
with pectin	.....	N. F.	..
with urea monophosphate	Monsanto Chem. Co.	Food	..

(*e*) Dried at 45° for one and one-half hours in circulating oven.
(*f*) Forced through a No. 12 sieve (U. S. Standard).
(*g*) Added lubricant and mixed thoroughly.
(*h*) Compressed on Stokes B-2 Rotary Tablet Machine.

Procedure No. 2

This procedure was the same as the above except that in step (*b*) the lactose was mixed with all but 5% of the disintegrating agent and in step (*g*) the lubricant and the 5% remainder of the disintegrating agent were added and mixed well.

Tablet Tests Used.—A Monsanto Hardness Tester was used to determine the hardness of the tablets since the hardness was a primary variable factor.

The tablets were tested for disintegration time immediately after manufacture and after a time interval of 500 hours.

The disintegration time was tested as outlined in the U. S. P. XIV on page 700. This test includes the fluid agitation of uncoated compressed tablets in water at 37° with a vertical stroke of 6 cm. at a rate of 30 cycles per minute. The test was considered complete when all the tablet material had passed through the retaining screen.

In order that results obtained from this official test may be considered on a comparative basis, it was necessary to extrapolate the tested times of disintegration to a standard. A standard hardness of 7 Kg. was chosen. It was found by testing hardness and times of disintegration that the agents exhibit a solubility-hardness ratio which, when plotted on quadrille-ruled graph paper, results in a curve. The curve was integrated for the necessary data. The extrapolations and integrations were used to collect the data for Tables II and III.

The tablets were exposed to a temperature of 4° and 45° for 500 hours and tested for disintegration time after allowing them to resume room temperature.

Results of Tables.—Results shown in Table II indicate that dried citrus pulp and powdered sponge used as disintegrating agents are more effective than many of the commonly used agents such as starch. Their disintegration time compares favorably with that of Bayer aspirin and Syntrogel.

Results shown in Table III indicate that aging of tablets at room temperature caused hardening in some cases but as a rule had little effect on hardness or on disintegration. Aging at reduced temperatures had no effect on hardness or disintegration time. However, it had an effect of removing any odors from the tablets. Aging of the tablets at elevated temperatures had a marked deleterious effect on hardness, stability, color, and disintegration time. The tablets became hard, discolored, and did not disintegrate.

TABLE II.—DISINTEGRATION RATE OF TABLETS IMMEDIATELY AFTER COMPRESSION USING VARIOUS AGENTS AS DISINTEGRANTS

Agent	Appearance of Tablets	Granulation Formula Used	Disintegration Time in Seconds — Procedure 1 — Tested Hardness, Kg.	Procedure 1 — Time	Procedure 1 — Time at 7 Kg. Hardness	Procedure 2 — Tested Hardness, Kg.	Procedure 2 — Time	Procedure 2 — Time at 7 Kg. Hardness
Control (no agent)	White	2	11	1440	880	..	..	..
Syntrogel	White	..	7	40	40	..	..	..
Bayer aspirin	White	..	4.5	11	20	..	..	..
Celloid 50	White, grainy	2	5	600[a]	600[a]	6	600[a]	600[a]
Bentonite	Tan	2	8	600[a]	600	5	480	520
Aveeno	White	2	3.5	120	200	3	120	200
Sponge	Tan	1	7	14	14	..	..	..
Citrus pulp	White, grainy	2	3	30	65	..	..	..
Pectin	White	1	6	260	270	6	260	270
Algin	White	2	5	600[a]	600[a]	7	600[a]	600[a]
Sodium alginate	White	2	3.5	600[a]	600[a]	3.5	600[a]	600[a]
Corn starch	White	2	7	180	180	7	150	150
Locust bean gum	White	2	4.5	90	140	5	100	140
Veegum	White	2	9	600[a]	600[a]	4.5	600[a]	600[a]
Tragacanth	White	2	5	600[a]	600[a]	9	600[a]	600[a]
Karaya gum	White, grainy	2	4.5	540	600	4.5	540	600
Methylcellulose, 100 c. p. s.	White	2	3.5	330	400	3.5	390	460
Methylcellulose, 4000 c. p. s.	White	2	4.5	150	200	3	120	210
C. M. C. 70	White	2	5	600[a]	600[a]	5.5	600[a]	600[a]
Magnesium peroxide	White	1	2.5	150	170	5	150	170
Sodium pyrophosphate peroxide	White	1	4	530	600[a]	4	390	600
Sodium carbonate peroxide	White	1	4	480	525	5	600	600
Citric acid with calcium carbonate	White	1	8	90	85	..	..	..
Pectin with calcium carbonate	White	1	3.5	105	185	..	..	..
Urea monophosphate with calcium carbonate	White	1	2	390	600[a]	..	..	..

[a] Showed no sign of disintegration after ten minutes by the U. S. P. test.

DISCUSSION

The effect of the addition of disintegrating agents in the normal manufacturing procedure for tablet making compared with the addition of a portion of the agent with the lubricant gave favorable results only with corn starch. With the other agents this procedure had little effect on time or rate of disintegration.

Comparison with two commercial samples showed that sponge and citrus pulp compare favorably with the slugged-type tablets of Bayer aspirin and Hoffman-La Roche's Syntrogel in rapidity and completeness of disintegration.

Tablets which did not appear to disintegrate after ten minutes contained disintegrating agents incapable of absorbing large quantities of water. On close examination it was found that a slimy, water-impervious layer of gum had formed on the exterior of the tablet. Thus any disintegration was improbable.

Among the peroxides tested, magnesium peroxide gave the best results. This type of disintegrating agent must be used in the absence of water and oxidizable material.

Of the effervescent-type tablets, good results were obtained with citric acid and pectin as the acid producing carbon dioxide from calcium carbonate.

Locust bean gum and Aveeno gave notable results. Corn starch—the tried, tested, and most widely used agent—gave favorable results. Oven predrying below 40° is necessary with corn starch. If dried too long so as to remove too much water or if exposed to too high a temperature, the efficiency of the agent is impaired (7).

Of special interest is the disintegration time of the two new agents, powdered sponge and dried citrus pulp. A very fast and complete disintegration was the result of the use of these agents.

The agent, dried citrus pulp, is nontoxic in pharmacological action. It does, however, result in a grainy tablet due to a slight amount of color and oils from the rinds of the fruits. It appears logical that citrus pulp is a better agent than pectin, which is derived from it, because as a raw product it contains tied-up unhydrolyzed sugars and celluloses which give it its action. The pulp may be decolorized or de-oiled by extraction with solvent, and possibly by absorptive methods.

Sponge was the best of the agents tested in this research. It caused very rapid and complete disintegration. However, a tan-colored tablet resulted from its use. Similar to bentonite, this may limit the use of untreated sponge in uncoated tablets (8); however, sponge can be bleached. More research must be done to establish the bleaching of this agent without impairing its powers as a disintegrating agent. Research on the toxicity of sponge showed that it is pharmacologically nontoxic. Further work is indicated here to establish its safe use. The theory of action of sponge as a disintegrating agent probably lies in its ability to hydrate, quickly, either by adsorption or by absorption into myriads of minute interstitial spaces of the sponge skeleton, thus swelling to a wet volume much greater than its dry volume. Such quick action along with the capacity of holding a large volume of liquid results in an effective disintegrating agent.

In Table II, the columns headed "Tested Hardness" and "Time" refer to the hardness and time of disintegration of the individual tablets as prepared. It was necessary to prepare the individual agents at varying hardnesses of tablets because of the nature

TABLE III.—EFFECT OF AGING AT ROOM TEMPERATURE, AT 4° C., AND AT 45° C. ON DISINTEGRATION TIME

	Disintegration (Sec.) at 7 Kg				Hardness	
Agent	0 Hr.	500 Hr. at 25° C.	500 Hr. at 4° C.	500 Hr. at 45° C.	Original, Kg.	500 Hr. at 45° C.
Control (no agent)	880	880	880	1400	11	14
Syntrogel	40	50	60	110	7	8.5
Bayer aspirin	20	20	20	24	4.5	7
Gelloid 50	600[a]	600[a]	600[a]	600[a, c]	6	10
Bentonite	520	600	600	600[f]	8	12
Aveeno	200[f]	200[f]	190	190[f]	3.5	3.5
Sponge	14	14	16	13	7	7
Citrus pulp	65	65[f]	50	65	3	3
Pectin	270	500	480	600	6	12
Algin	600[a]	600[a]	600[a]	600[a]	7	7
Sodium alginate	600[a]	600[a]	600[a]	600[a]	3.5	7
Corn starch	150	150	150	300	7	9
Locust bean gum	140	140	140	140	4.5	6
Veegum	600[a]	600[a]	600[a]	600[a]	4.5	7
Tragacanth	600[a]	600[a]	600[a]	600[a, b, c]	5	9
Karaya gum	600[f]	600[f]	600	600[b, c]	4.5	4.5
Methylcellulose, 100 c. p. s.	400	420	400	420[f]	3.5	7
Methylcellulose, 4000 c. p. s.	200	180	140	120	3	3
C. M. C. 70	600[a]	600[a]	600[a]	600[a, c]	5	5
Magnesium peroxide	170	180	200	200	5	12
Sodium pyrophosphate peroxide	600	600	600	600[f]	4	4
Sodium carbonate peroxide	525[f]	525[f]	525	525[b, c]	4	11
Citric acid with calcium carbonate	85	75	90	85[c, d]	8	8
Pectin with calcium carbonate	185	95	100	110	3.5	3.5
Urea monophosphate with calcium carbonate	600	600[a, c]	600[a]	600[a, b, c]	2	15

[a] Showed no sign of disintegration after ten minutes by U. S. P. test.
[b] Denotes brown coloration of tablets.
[c] Denotes development of odor in tablets.
[d] Denotes development of speckled appearance in tablets.

of the agents; i. e., their relative ability to compress, their tendency to produce more or less "fines" per granulation, their relative hardness caused by the binding agent, and the type of granulation formed. Therefore, to obtain results of a comparative nature, the disintegration times were extrapolated from individual hardnesses to a hardness of 7 Kg. by the method previously discussed.

The effect of aging the tablets brought forth some data that influence the choice of disintegrating agent. The action of aging of tablets at room temperature resulted in no appreciable effect over a period of 500 hours. Pectin was the only agent that was in any way affected. Hardness may develop on storage of tablets in which gums such as acacia, pectin, etc., are used for granulating or disintegrating (9). No other changes in hardness, color, odor, or disintegration time were noted.

The exposure of the prepared tablets to 4° for 500 hours showed that cold had no bad effect on disintegrating time or hardness. It will be noted that a slight decrease in disintegration time resulted. No effect on color or appearance of the tablets was noted. The cold aging of tablets having odors seems to be indicated industrially. In these tests, tablets which had an odor on manufacture were leached of that odor by subjection to 4° for 500 hours in sealed containers.

Heat aging, 45° for 500 hours, had a marked and deleterious effect. Color, odor, hardness, and disintegration time were affected. With many agents the color changed from white to brown due to the reaction of the ingredients with heat probably causing decomposition, oxidation, and caramelization. Offensive odors developed in many of the tablets that changed color, and also in many in which no color change was noted.

A greater percentage of the affected agents were those that that were exposed to heat aging. In many cases the hardness of the tablets doubled. This is probably due to fusing of materials, cementation, hardening of the binder, loss of moisture and volatile substances, and chemical decomposition of the disintegrating agent and other ingredients of the tablet. Since hardness is a primary variable factor of disintegration time, the time rate of disintegration of the tablets was greatly affected by the heat aging. The time of disintegration increased.

SUMMARY

A comparative study of 22 tablet disintegrating agents was carried out. New, as well as commonly used, agents were tested, and favorable results were shown by two of these new agents. Dried citrus pulp and powdered dried sponge compare favorably as tablet disintegrants with the best agents in use by pharmaceutical manufacturers.

The effect of aging tests established a new criteria by which tablet disintegrators must be selected. Aging at room, elevated, and reduced temperatures for a measured time interval indicated that tablets are markedly affected by heat. Cold had little effect on tablets. Aging at room temperature showed effect only with a certain type of agent which forms a harder tablet over a period of time.

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Teachers' Seminar on Pharmaceutical Chemistry Announced

The Teachers' Seminar on Pharmaceutical Chemistry will be held in Ann Arbor, Mich., July 7–12, 1952. The College of Pharmacy of the University of Michigan was selected to be the host institution by the Executive Committee of the American Association of Colleges of Pharmacy.

This Seminar will be the fourth to be sponsored by the A. A. C. P. The funds for the previous seminars and for this one are provided by the American Foundation for Pharmaceutical Education.

Dr. Tom D. Rowe, chairman of the committee for planning the Seminar, has announced that preliminary plans have been made for the program, which will include all branches of pharmaceutical chemistry. It is expected that teachers from most of the Colleges of Pharmacy will be in attendance. The emphasis throughout the program will be on teaching methods, course contents, and new developments. Arrangements have been made to provide accommodations for those attending in one of the new dormitories at the University of Michigan.

Pharmacological Actions of *Paeonia officinalis*[*,†]

By DUANE G. WENZEL[‡] and ALBERT R. HASKELL[§]

A crude, dealcoholized alcoholic extract of peony root was screened for pharmacological activities. It was found to produce digitalis-like action on the frog's heart, stimulation of the uterus of the rat, and depression of the intestine of the rat and rabbit. Minor lowering of the blood pressure was produced in the rat. The extract was ineffective in combating electroshock and Metrazole convulsions and lacked analgesic activity.

The common garden peony, *Paeonia officinalis*, is a member of the family *Ranunculaceae*. A native of Southern Europe and Western Asia, it is now found throughout the temperate zone of the Northern Hemisphere. Hippocrates and Galen were among the ancients recommending parts of the plant as treatment for diseases ranging from epilepsy to the malfunctions of the female genitourinary system (1). An alkaloid, peonine, was stated by Rochbrune (1) to be similar in its actions to aconitine. Holste (2) reported an alkaloid that stimulated the uterus and decreased the coagulation time of the blood. The hemostatic principle has since been identified as peonol, an aromatic lactone (3). Sugihara (4) noted that the root produced hyperglycemia in the rabbit.

The present preliminary study was undertaken to screen the extract of the peony root for the more significant reported activities and historical uses.

EXPERIMENTAL

Preparation of Solutions.—The solid, dealcoholized, alcoholic extract representing 15% by weight of the dried root of *Paeonia officinalis* Linne var. *number 4 pink* was the test material. Solutions were prepared for intact animal and isolated organ studies by suspending the extract in the fluid indicated. In the experiments upon the heart and blood pressure the suspended material was removed by filtration. The filtered solution is referred to as the "prepared extract." In each instance the *p*H of the drug solution in the proper physiological salt solution was adjusted to that of the original medium.

Toxicity.—The LD_{50} for the extract was determined by intraperitoneal administration in order to establish dosage levels for *in vivo* experiments. Male, albino, Swiss-strain mice and male Sherman-Wistar strain rats were used according to accepted procedures. The LD_{50}'s were 795 and 700 mg./Kg., respectively. The data were analyzed by the statistical procedure of Litchfield and Wilcoxon (5). Toxic symptoms did not appear until after twenty-four hours. These were primarily irritability and partial paralysis. Death occurred between forty-eight and fifty-six hours after the injection. Gross pathological examination revealed the adrenals to be highly vascular and the heart stopped in systole. Oral toxicity was not evidenced in rats or mice with doses approaching the limit of the stomach's capacity.

Uterus.—The uterine activity of the total extract in nutrient Tyrode's solution (6) was determined at a concentration of 1:1,000 on the gravid and nongravid excised uterus of the Wistar-strain rat. Rats were used as a source of uterine tissue since the cycle of contraction and relaxation is more rapid than either the guinea pig or the rabbit (7) and because it shows a greater uniformity of response (8). As shown in Fig. 1, the drug produced an immediate increase in the amplitude of contraction which returned to normal in five to twenty-five minutes. Washing with fresh Tyrode's solution after the return to normal caused a marked tonicity persisting for a minimum of ninety minutes. Qualitative results for the pregnant and nonpregnant uteri did not differ.

Fig. 1.—Effect of *Paeonia officinalis* extract on nonpregnant rat's uterus. Timed at five-minute intervals.

Intestine.—A modification of the method of Sollmann and Rademaekers (9) was employed. The peristaltic pressure changes were recorded by a 10-cm. segment of rabbit's intestine. Figure 2 indicates the effect of the drug in concentration of 1:1,000 producing a definite relaxation followed by normal rhythm after washing. The depressant activity was verified by the method of Magnus (10) with intestinal strips from the rat.

Frog's Heart.—Cardiac activity was determined on *Rana pipiens* with a Greene perfusion cannula in the sinus venosus. A 1:1,000 concentration of the

* Received October 22, 1951, from the School of Pharmacy, University of Kansas, Lawrence.
Abstracted from a thesis submitted to the Graduate School of the University of Kansas by Albert R. Haskell in partial fulfillment of the requirements for the degree of Master of Science.
† The peony roots used in this study were supplied by Wild Brothers Nursery Co. and Gilbert H. Wild and Son, Sarcoxie, Mo.
‡ Assistant Professor of Pharmacy, School of Pharmacy, University of Kansas, Lawrence.
§ Present address: Instructor in Pharmacology, College of Pharmacy, University of Florida, Gainesville.

Fig. 2.—Effect of *Paeonia officinalis* extract on rabbit's intestine. Timed at two-minute intervals.

"prepared extract" in Howell's solution was used to perfuse the heart. The diastolic size of the heart was reduced while the systolic size remained normal until shortly before the digitalis-like systolic standstill. Figure 3 shows this effect and recovery after washing.

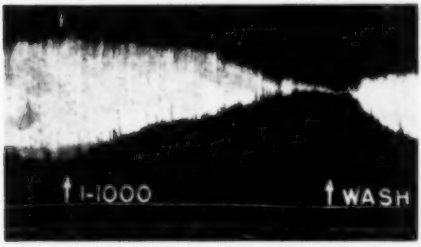

Fig. 3.—Effect of *Paeonia officinalis* extract on frog's heart. Timed at one-minute intervals.

Effect on the Blood Pressure.—Rats were anesthetized by the intraperitoneal administration of pentobarbital sodium (50 mg./Kg.). The carotid artery was cannulated with a 20-gauge needle filled with a solution of heparin in normal saline. This in turn was attached to the pressure head of a Technitrol C-R Lilly Manometer which controlled the writing beam of a Hathaway Mechanical Oscilloscope. One milliliter of the "prepared extract," equivalent to 160 mg./Kg. of the total extract, was injected into the jugular vein. A transitory fall in blood pressure was produced. As shown in Fig. 4, the administration of 4 μg. of epinephrine immediately following the return to normal caused no reduction in blood pressure, indicating the absence of adrenolytic action.

Analgesia.—The hot-plate method of Woolfe and MacDonald (11) utilizing albino Swiss-strain mice was used to determine the activity. A dose of 600 mg./Kg., representing 75% of the LD_{50}, was employed. A significant degree of analgesia was not shown.

Antiepileptic Activity.—Swinyard's technique (12) using Metrazol and maximal electroshock tests was employed. The electroshock seizures were produced by a half-wave rectified 60-cycle alternating current apparatus. The extract was administered intraperitoneally to male Sherman-Wistar strain rats in a dose of 525 mg./Kg. (75% of the LD_{50}). A current of 150 ma. was delivered for 0.2 second by ear electrodes. The Metrazol was administered in a dose of 70 mg./Kg. Abolition of the tonic extensor component of the resultant convulsion was taken as the end point of the test. Tests were conducted at half-hour intervals for two hours and then every two hours for a total of twelve hours. Further tests were made at twenty-four hours. Prolonged testing was carried out to detect any delayed activity as evidenced by the toxicity. The pattern of the convulsions produced was not altered in any animal at any of the times tested, indicating absence of antiepileptic activity.

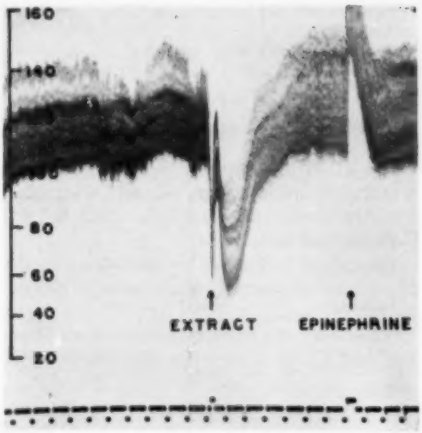

Fig. 4.—Effect of *Paeonia officinalis* extract (160 mg./Kg.) on the rat's blood pressure. Timed at one-minute intervals.

DISCUSSION

Paeonia officinalis produces a marked stimulation of the gravid and nongravid rat's uterus. The effect is an increase in the amplitude of contraction. A bizarre secondary effect of prolonged tonicity is produced when the uterus is washed with the original bath fluid. A possible explanation for the secondary increase in uterine tonicity may be found in the depression produced with the intestine. It may be postulated that the initial lack of uterine tonicity is caused by this depressant principle.

The drug causes a marked digitalis-like effect on the frog's heart in concentration of 1:1,000. The fatal dose for rats and mice likewise stopped the heart in systole. These results are not in accord with those of Rochbrune (1) who indicated an aconitine-like action for a crude extract of the entire plant and for three crystalline substances obtained from the roots, seeds, and flowers. He designated all three compounds as peonine. His crude extract as well as the peonines stopped the guinea pig and frog hearts in diastole on subcutaneous and intralymphatic injections, respectively.

A transient fall in blood pressure is produced in the rat. It was shown to be nonadrenolytic in nature. Blood pressure recordings were not made with other species.

Neither analgesic nor antiepileptic activity could be demonstrated.

The cardiac and uterine stimulant actions appear sufficiently significant to warrant further investigation.

SUMMARY

The LD_{50}'s of the peony root extract administered intraperitoneally are 795 and 700 mg./Kg., respectively, for mice and rats. Oral toxicity could not be demonstrated.

The extract produces a twofold action on the rat uterus and depresses the intestine of the rat and rabbit.

It has a digitalis-like action on the frog's heart. The drug causes a moderate depression in the blood pressure of the rat and has no adrenolytic activity.

Analgesic and antiepileptic activity are absent.

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A Comparison of the Effects of Anthallan, Pyribenzamine, and Epinephrine on Smooth Muscle*,†

By REBECCA C. HELLERMAN and LLOYD W. HAZLETON

The antispasmodic properties of Anthallan on reactions similar to those involved in asthma have been studied by the tracheal chain method of Castillo and deBeer, on guinea pigs sensitized to egg white. Comparisons have been made on the effective concentrations of Anthallan, epinephrine, and Pyribenzamine necessary to relax histamine contractions of the ileum and tracheal chain. Comparisons have also been made on the effective concentrations of Anthallan, epinephrine, and Pyribenzamine necessary to relax allergic (egg white) contractions of the ileum and tracheal chain. The results of these studies show that epinephrine is equally effective against histamine and allergic contractions of the ileum and at higher concentrations is equally effective against histamine and allergic contractions of the tracheal chain. Pyribenzamine is more effective than Anthallan against histamine contractions of the ileum and the tracheal chain. However, Pyribenzamine and Anthallan were equally effective against the allergic contractions of the ileum, but Anthallan was more effective than Pyribenzamine against the allergic contraction of the tracheal chain.

ANTHALLAN has been used with some success in the treatment of bronchial asthma, vasomotor rhinitis, and different types of dermatitis (1–4). Hazleton (5) presented a summary of the reports on the pharmacological and toxicological properties of Anthallan, showing that the clinical results may be independent of the mild antihistaminic action. Haley and Andem (6), in studies of antihistamines upon the mammalian capillary bed, showed that Anthallan was a potent local vasoconstrictor.

In view of these various results, not all of which appear to be consistent, the present studies were undertaken to evaluate the potency of Anthallan against allergic and histamine-induced contractions of guinea-pig ileum and tracheal chain. The guinea-pig tracheal chain method of Castillo and deBeer (7) and Castillo (8) is particularly well adapted for the study of bronchial constriction and of bronchial dilator drugs.

Castillo (9) showed that the antihistaminic drugs to not cause anaphylactic contractions of the tracheal chain. However, these allergic contractions are relaxed by the bronchial dilators such as aminophylline, papaverine, and epinephrine. The present studies were designed to compare the effective concentrations of Anthal-

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Presented to the Scientific Section, A. PH. A., Buffalo meeting, August, 1951.
† Acknowledgment is made of a grant from the Medico-Chemical Corporation of America in support of these investigations.

lan, epinephrine, and Pyribenzamine (in the form of the soluble hydrochlorides) for relaxing histamine contractions of the ileum and tracheal chain. Comparisons have also been made of the effective concentrations of Anthallan, epinephrine, and Pyribenzamine necessary to relax anaphylactic (egg white) contractions of the ileum and tracheal chain.

EXPERIMENTAL

Nonsensitized animals were used for obtaining histamine contractions of the ileum and tracheal chain. In the allergic studies the method used was that previously described by Hellerman and Hazleton (10) for guinea pigs sensitized to egg white. Guinea pigs were sensitized by injecting intraperitoneally 5.0 cc. of 25% egg white in normal saline. These animals were sacrificed by a blow on the head fourteen to twenty days after injection. The ileum was suspended in Locke's solution maintained at a constant temperature of 37°. The lever ratio was 2:1. Egg white added to the bath caused a strong allergic contraction.

The tracheal chains were suspended in Hastings-Van Dyke solution, with a lever ratio of 12:1. Egg white added to the bath caused a strong allergic contraction.

Various concentrations and doses of Anthallan hydrochloride, epinephrine hydrochloride, and Pyribenzamine hydrochloride were used in order to establish the doses which will relax a contraction to the baseline. Table I presents these effective doses. Representative sections from the records showing the relaxation of contractions are also presented.

Figure 1 shows the relaxation of a submaximal histamine contraction of the guinea-pig ileum by these three drugs. It will be noted that Pyribenz-

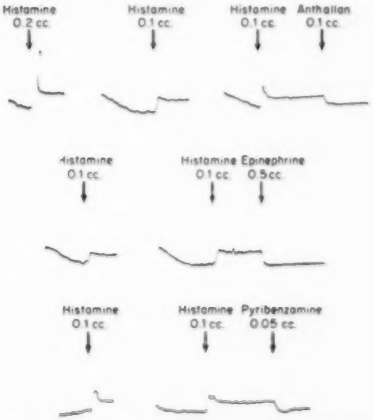

Fig. 1.—Effect of Anthallan HCl (10 mg./cc.), epinephrine HCl (0.01 mg./cc.), and Pyribenzamine HCl (0.05 mg./cc.) on histamine (0.01 mg./cc.) contractions of isolated guinea-pig ileum.

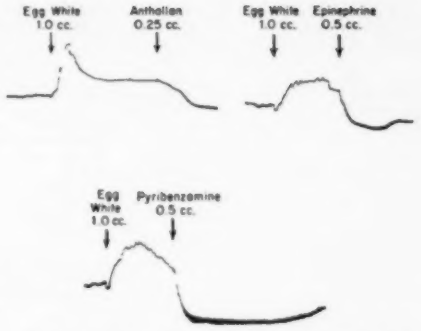

Fig. 2.—Effect of Anthallan HCl (10 mg./cc.), epinephrine HCl (0.01 mg./cc.), and Pyribenzamine HCl (5.0 mg./cc.) on egg white contractions of isolated guinea-pig ileum.

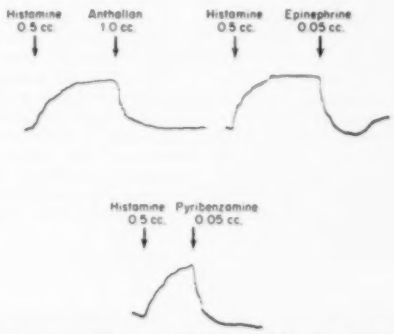

Fig. 3.—Effect of Anthallan HCl (10 mg./cc.), epinephrine HCl (1.0 mg./cc.), and Pyribenzamine HCl (5.0 mg./cc.) on histamine (1.0 mg./cc.) contractions of guinea-pig tracheal chain.

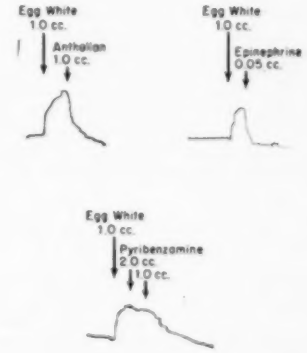

Fig. 4.—Effect of Anthallan HCl (10 mg./cc.), epinephrine HCl (1.0 mg./cc.), and Pyribenzamine HCl (10 mg./cc.) on egg white contractions of guinea-pig tracheal chain.

TABLE I.—SUMMARY OF EFFECTIVE CONCENTRATIONS OF ANTHALLAN, EPINEPHRINE, AND PYRIBENZAMINE AGAINST HISTAMINE AND EGG WHITE ALLERGIC CONTRACTIONS OF GUINEA-PIG ILEUM AND TRACHEAL CHAIN

Muscle	Spasmodic	Material	Concentration, Mg./Cc.	Dose, Cc.	Total, Mg.	Final Dilution
Ileum	Histamine		0.01	0.1	0.001	1:100,000,000
		Anthallan	10.0	0.1	1.0	1:100,000
		Pyribenzamine	0.05	0.05	0.0025	1:40,000,000
		Epinephrine	0.01	0.5	0.005	1:20,000,000
	Egg white		Undiluted	1.0		1:100
		Anthallan	10.0	0.25	2.5	1:40,000
		Pyribenzamine	5.0	0.5	2.5	1:40,000
		Epinephrine	0.01	0.5	0.005	1:20,000,000
Tracheal chain	Histamine		1.0	0.5	0.5	1:200,000
		Anthallan	10.0	1.0	10.0	1:10,000
		Pyribenzamine	5.0	0.05	0.25	1:400,000
		Epinephrine	1.0	0.05	0.05	1:2,000,000
	Egg white		Undiluted	1.0		1:100
		Anthallan	10.0	1.0	10.0	1:10,000
		Pyribenzamine	10.0	3.0	30.0	1:3,300[a]
		Epinephrine	1.0	0.05	0.05	1:2,000,000

[a] Atypical relaxation.

amine is the most potent. Figure 2 shows the relaxation of egg white contractions of the guinea-pig ileum. Epinephrine is the most potent, whereas Anthallan and Pyribenzamine, while considerably less potent, are equal to each other. Figure 3 shows the relaxation of histamine contractions of the tracheal chain. Epinephrine is the most potent and Anthallan the least potent. Figure 4 shows that epinephrine is the most potent and Pyribenzamine the least potent against egg white contractions of the tracheal chain. It will be noted that the Pyribenzamine relaxation is atypical in that it is very slow and incomplete.

DISCUSSION

Epinephrine is the most potent antispasmodic against all types of reactions studied except the histamine-induced contraction. Epinephrine is effective at the same concentration against histamine and egg white contractions of the ileum and, at higher concentrations, is equally effective against histamine and egg white contractions of the tracheal chain. Pyribenzamine is more effective than Anthallan against histamine contractions of the ileum and the tracheal chain; however, Pyribenzamine and Anthallan are equally effective against allergic contractions of the ileum. Anthallan is more effective at a lower concentration than Pyribenzamine against allergic contractions of the tracheal chain.

These results indicate that in allergic contractions of the tracheal chain the potency of Anthallan is markedly increased in relation to that of epinephrine and Pyribenzamine. This is an example of the inherent difference between allergic and histamine reactions, as pointed out by Castillo (9), and a further demonstration of the utility of the tracheal chain method for evaluation of bronchodilator drugs.

SUMMARY

1. Epinephrine is equally potent against histamine and allergic contractions.

2. Pyribenzamine is more potent than Anthallan against all histamine contractions.

3. Anthallan is equal to or more potent than Pyribenzamine against all allergic contractions.

4. The action of Anthallan against allergic contractions of the tracheal chain appears to be relatively specific.

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Book Reviews

Medicinal Chemistry. Vol. II. By ALFRED BURGER. Interscience Publishers, Inc., New York, 1951. xv + 506 pp. 16 x 23.5 cm. Price $10.

This is the second of two volumes of a treatise on medicinal chemistry by Alfred Burger of the Department of Chemistry of the University of Virginia. The first volume, published in 1951, was described and reviewed earlier [THIS JOURNAL, **40,** 417(1951)]. The second volume is a continuation of the first, in which the major part is devoted to drugs used in the chemotherapy of diseases caused by pathogenic organisms. In addition, however, chapters on the chemistry of the hormones and their uses in medicine, chemotherapy, and theories of metabolite antagonism are included. The chapter on theories of metabolite antagonism is written with unusual clarity and conciseness.

The chapters on dyestuffs in chemotherapy, the sulfonamide drugs, antimalarials, metal-free drugs used in tropical diseases, antibiotics, antifungal agents, anthelmintics, organometallic chemotherapeutic compounds of arsenic, antimony and bismuth, and antiseptics, are all handled adequately and with Professor Burger's customary skill. In the several fields covered, the available literature relating thereto was reviewed completely up to 1951. Thus with the publication of the second volume of *Medicinal Chemistry,* a modern, usable, and essentially up-to-date treatise on the subject has been made available.

One of the greatest technical difficulties in the preparation of a book of the type of *Medicinal Chemistry* is the preparation of an adequate subject index. The subject index to Volumes I and II represents a real accomplishment. This index, consisting of 57 pages, includes a sufficient number of entries so that no difficulty is experienced in quickly locating information on any phase of the subject covered. Professor Burger's book should have ready acceptance, both as a textbook and a reference, by all who are interested in any phase of medicinal chemistry.

Fluorine and Its Compounds. By R. N. HASZELDINE and A. G. SHARPE. John Wiley & Sons, Inc., New York, 1951. 153 pp. 11 x 17 cm. Price $1.75.

This little book is a convenient review of the history and development of the chemistry of fluorine up to 1950. The authors compiled this concise monograph during the same period that a much more comprehensive treatise was being prepared by C. Slesser and S. R. Schram [reviewed in THIS JOURNAL, **41,** 58(1952)]. Dr. Haszeldine's intimate association with the subject matter covered in his text is indicated by the fact that 13 of the 91 references are to his publications. An index is appended.

A Study of Antimetabolites. By D. W. WOOLLEY. John Wiley & Sons, Inc., New York, 1952. xiii + 269 pp. 15.5 x 23.5 cm. Price $5.

The author of this book, Dr. D. W. Woolley of the Rockefeller Institute for Medical Research, has presented a logical, modern, and thoroughly intelligible account of experimental results which have been accumulated during the past ten or fifteen years on antimetabolites. A knowledge of this subject may contribute to an understanding of many unanswered problems in pharmacology, chemotherapy, and biochemistry. For this reason the book should be of special interest to pharmaceutical chemists and pharmacologists.

The introduction alone serves admirably to orient the reader to the subject of antimetabolites. The book presents a thorough discussion of the antagonisms between structurally related compounds and original concepts concerning the nature of antimetabolic action, and points the way to the application of that concept to solve problems in chemotherapy, biochemistry, and pharmacology. Among other features, the book includes a tabulation of antimetabolites acting against many constituents of living cells. The value of the book is enhanced by a bibliography of 500 references to the original literature. It is also provided with an index of structural formulas and an adequate subject index. The printing and binding are of excellent quality.

Substances Naturelles de Synthese. Vol. III. By LEON VELLUZ. Masson et Cie, Paris, 1951. 156 pp. 16 x 23.3 cm. Price 1500 fr.

The same authors (J. Mathieu, A. Petit, and P. Poirier) have collaborated with Dr. Velluz in the three volumes of this series and the same style is carried on. The review of the first two volumes [THIS JOURNAL, **40,** 534(1951)] described the arrangement of the text.

Detailed descriptions of the important methods of syntheses with summaries of other methods are given for the following compounds: DL-glutamic acid, DL-dihydroxyphenylalanine (DOPA), estradiol, DL-ornithine, and thiamine. The syntheses of equilenine, estrone, and oxythiamine are discussed.

Reactions involving the thiazole ring, especially the mono- and dimolecular condensations, and particularly the benzothiazoles, are discussed. Reaction schemes and references are tabulated. Methods of syntheses of amino-acids are discussed and the reactions and references are tabulated.

The procedures used in the determination of melting points and the practical utilization of this physical property are discussed.

A more detailed table of contents giving the intermediate compounds in the detailed syntheses, in addition to author and subject indexes, is appended.

This series of books should be available to everyone interested in the synthesis of organic compounds.

The Phosphatides. By HAROLD WITTCOFF. Reinhold Publishing Corporation, New York, 1951. xviii + 564 pp. 16 x 23.5 cm. Price $10.

The Phosphatides is the 112th of the monograph series sponsored by the American Chemical Society. The printing and binding are of the same type as that used in previously published books of the A. C. S. series of chemical monographs. According to the author, "The lack of a modern text in English on phosphatide chemistry has provided the inspiration for the present volume." That inspiration has resulted in a new book offering a logical survey of every conceivable phase of phosphatide chemistry. The nomenclature, isolation, proof of structure, properties, synthesis, physical chemistry, enzymic relationships, analysis, sources, biochemistry, medical aspects, metabolic relationships, and commercial utilization of phosphatides have been described and interpreted. An important feature of the book is that recent advances in phosphatide chemistry, based on a literature survey extending through 1951, are included.

The book is completely documented with references to the original literature. These references are collected and printed at the end of each major division of the book. The book is divided into six parts, each part consisting of an adequate number of logically arranged chapters to cover the several subjects. In Part I, the chemistry of the phosphatides is discussed, and in Part II, the procedures and analytical determination are described. In Part III, the phosphatides from plant sources are adequately treated, and Part IV is devoted to the phosphatides from animal sources. The biochemistry and physiology of the phosphatides are described in Part V, and Part VI treats the industrial aspects of these important substances. The book contains much information of interest to pharmaceutical chemists, and is recommended as an excellent reference to any phase of phosphatide chemistry.

Hormones: A Survey of Their Properties and Uses. Published by direction of the Council of the Pharmaceutical Society of Great Britain. The Pharmaceutical Press, London, 1951. xii + 220 pp. Illustrated. 14.5 x 22.8 cm. Price 35*s*.

The material in this book is selected to provide an account of those hormones and endocrine glands which have well-defined pharmacological effects and therapeutic applications. Closely related substances such as the artificial hormones and antihormones are included. The pineal and thymus glands, and some hormones which do not meet the admittance requirements are omitted. The term "artificial hormones" refers to substances with hormonal activity that are produced only in the laboratory.

The excellent text begins with an historical account of the development of the science of endocrinology to the isolation and synthesis of active principles, and continues with descriptions of the physiology of the glands, including their functions and the effects of dysfunction. The importance and limitations of replacement therapy are indicated. The hormones are divided into two groups according to their chemical nature as steroid and nonsteroid derivatives. The chapter on the chemistry of the steroid hormones gives a brief review of the general chemistry of steroids and it also includes the artificial hormones, which are related chemically or physiologically to certain of the steroid hormones.

The contributors and the chapters for which they are responsible are: H. E. Dale, Physiology and Actions and Uses; C. W. Emmens, Standardization; D. H. Hey, Chemistry; and T. D. Whittet, Pharmacy. The chapter on standardization describes the techniques and values of the methods of biological assay. It is pointed out, however, that once the production of the hormones as pure chemical substances is achieved, there is no object in continuing to use biological assay methods.

The hormones are divided for pharmaceutical purposes into two groups: those in which the active principle is of unknown chemical constitution, and those for which the information is available. The pharmacist's chief concern with the former is preservation of potency and storage. With the latter group there is scope for the application of pharmaceutical skill in presenting the active principles in the most suitable form for the particular purpose required in addition to insuring maximum activity and stability. The products recognized in the British Pharmacopoeia are discussed, and a list of commercial products available in Great Britain is included. The manufacturers and their addresses in Great Britain are also listed.

Bibliographies are given at the end of each chapter. An inclusive bibliography divided into general and individual endocrine gland sections, and a general subject index are added.

This book is a splendid contribution in this field and should be available to students, chemists, physicians, and pharmacists.

Accepted Dental Remedies. 17th ed. Council on Dental Therapeutics, American Dental Association, Chicago, 1952. xvi + 211 pp. 16 x 20.5 cm. Price $1.50.

Accepted Dental Remedies is published annually by the American Dental Association, and is designed to serve as a handbook on dental therapeutics. A casual examination of the book reveals few changes from one year to the next, but a careful perusal of each new edition discloses the fact that it differs considerably from the preceding one, and illustrates clearly that the status of dental products is undergoing constant change. The 1952 edition conforms to the changes incorporated in U. S. P. XIV and N. F. IX. In addition to containing a list of therapeutic products acceptable to the Council on Dental Therapeutics, it also states the provisions for acceptance of products by the Council. In the course of the latest revision a notable improvement has resulted from a rearrangement involving the incorporation in one chapter of all considerations relating to the disinfection of dental instruments. Of pharmaceutical interest are the formulas and tables and the section on symptoms and treatment of acute poisoning.

The design of the 1952 *Accepted Dental Remedies* is essentially the same as that followed during the past four or five years. The book is provided with an index to distributors of accepted products, and an unusually complete general index. The typography and binding are both excellent, but the paper cover seems a bit inadequate for such an otherwise fine book.

Chemistry of Carbon Compounds. Vol. I, Part A. Edited by E. H. RODD. Elsevier Publishing Co., Houston, Tex., 1951. xxi + 777 pp. 16 x 23.3 cm. Subscription price $18; list price 15% higher.

This book has been written and published to fill the need for an up-to-date reference book, intermediate in size between an encyclopedia and an advanced textbook on organic chemistry. It is intended to serve as a successor to Richter's *Organic Chemistry*, a reference book that has been used by several generations of organic chemists, but which, during recent years, has not been kept up to date.

Volume I of *Chemistry of Carbon Compounds* includes a general introduction, followed by a series of chapters on aliphatic compounds. The general introduction includes a number of chapters treating the physical properties of carbon compounds, and certain aspects of physical chemistry having special significance to organic chemistry. Stereochemistry and reaction mechanism are stressed. In Volume I, devoted to aliphatic compounds, the original style of presentation of Richter's *Organic Chemistry* has been retained, and the Richter classification of organic compounds is employed essentially in its original form. Volume I is adequately documented with selective references to the original literature with the citation of review articles and summaries especially stressed. The complete project anticipates the publication of four additional volumes before the work can be considered complete.

Volume II will cover the aliphatic compounds; Volume III, the aromatic compounds; Volume IV, heterocyclic compounds; and Volume V will be devoted to miscellaneous compounds and a general index.

Volume I includes an unusually adequate index which appears to be quite sufficient for the quick location of information on the aliphatic compounds covered. *Chemistry of Carbon Compounds* is edited by Dr. E. H. Rodd, who is assisted by a group of advisors including such well-known British chemists as Sir Robert Robinson, J. W. Cook, R. D. Haworth, Sir Ian Heilbron, E. L. Hirst, and A. R. Todd. Contributors to Volume I are also well-known British organic chemists, and each is an expert in the specialized field for which he assumed responsibility. Thus the general quality and accuracy of the successor to "Richter" seems to be assured.

When the five volumes are completed, *Chemistry of Carbon Compounds* should occupy a position comparable to that held for so many years by Richter's *Organic Chemistry*.

The Lemon Fruit: Its Composition, Physiology, and Products. By ELBERT T. BARTHOLOMEW and WALTON B. SINCLAIR. University of California Press, Berkeley, Calif., 1951. xi + 163 pp. Illustrated. 16 x 24.3 cm. Price $4.50.

Anyone seeing only the title of this book might think it was a piece of promotional literature compiled by a fruit growers' association, to increase the popularity of the lemon for culinary, beverage, industrial, and medicinal uses. Such an opinion, however, would be dispelled upon subjecting the book to even a casual examination. The authors, one a plant physiologist, and the other a biochemist in the Experiment Station at Riverside, Calif., have written an interesting and useful book in which they have summarized an enormous amount of general and technical knowledge concerning the lemon.

The first chapter, entitled General Information, discusses the origin, history, principal varieties, and chemical changes which occur during the growth and maturation of the lemon. The second and longest chapter covers the composition and physiology of the fruit. In the third chapter the products of the lemon fruit and their uses are discussed. In this chapter the reader learns many surprising facts about the utilization of the lemon in the production of many and varied products used in beverages, perfumes, drugs, and as a food for stock, recovered from waste pulp resulting from the processing of lemons.

The book should be interesting not only to fruit growers, processers, shippers, food technologists, and other research workers, but also to the general reader. The book is provided with an extensive bibliography, an author index, and a subject index.

Formulary and Therapeutic Guide. Compiled by the Formulary Committee of the New York Hospital. Appleton-Century-Crofts, Inc., New York, 1951. xv + 355 pp. 12.5 x 19.5 cm.

The 1951 edition of the Formulary of the New York Hospital, a book which has been published since 1816, is undoubtedly a valuable aid and guide to those who carry out the policies of that center of medical care. It contains much useful information for practitioners of medicine and pharmacy wherever they might be actively engaged. It presents in concise form the description, therapeutic use and mode of administration, precautions to be observed, and the amounts to be dispensed of various selected materials. The monographs include U. S. P., N. F., N. N. R., proprietary, and other unofficial drugs and special formulations for compounded preparations.

It is interesting to note that the New York Hospital has not been affected by the present difficulties encountered in the use of Phenolated Calamine Lotion N. F. IX prepared from Calamine Lotion U. S. P. XIV. The Formulary Committee prefers the N. F. Neocalamine to the U. S. P. Calamine and prescribes the use of neocalamine liniment, lotion, and phenolated lotion that resemble the N. F. products with some variations. It is surprising that neocalamine has not been more widely utilized in general dermatologic therapy. Medical supplies and prescription accessories such as hypodermic needles, bandages, graduated medicine droppers, plain droppers, plasma-withdrawal flasks, etc., are included.

All narcotic drugs and preparations are identified with the word "narcotic" in parentheses beside the title, and it is printed in the same type and face as the title. The word "hypnotic" is similarly used to designate drugs and preparations in that group.

The uncertain meaning of "teaspoonful" is recognized by indicating the metric equivalent as 4–5 cc. Since the Formulary is primarily for the guidance of the hospital staff, either 4 cc. or 5 cc. should be a teaspoonful, and the procurement officer of the hospital could purchase volume-tested teaspoons accordingly. This would enable uniform dosage calculations for teaspoonful doses.

The book does not have a general index but all monographs are cross-indexed in the text, and a therapeutic index is appended.

Interim Revision Announcement

PHARMACOPEIA OF THE UNITED STATES FOURTEENTH REVISION

Three revisions of U. S. P. XIV have been approved, effective as indicated below:

PROTAMINE ZINC INSULIN INJECTION

Protamine Zinc Insulin Injection, page 296—Effective immediately, change the last sentence of the section on *Labeling* to read:

The outside labeling of each retail package shall also state a date of expiration which must not be later than 24 months after the immediate container was filled.

Official and Released December 7, 1951

NEOSTIGMINE METHYLSULFATE INJECTION

Injections, Third U.S.P. XIV Supplement, page 13, section on *Packaging and Storage*—Effective immediately, delete Neostigmine Methylsulfate Injection from the list of items which shall be packaged in single-dose containers and add it to the list of items which may be packaged in either single-dose or multiple-dose containers.

Official and Released February 8, 1952

VITAMIN B_{12} ACTIVITY ASSAY

Vitamin B_{12} Activity Assay, Third U.S.P. XIV Supplement, page 15, section on *Culture Medium*—Add the following sentence:

Cool as rapidly as possible to avoid color formation resulting from overheating the medium.

Vitamin B_{12} Activity Assay, Third U.S.P. XIV Supplement, page 15, section on *Inoculum*—Change the next-to-last sentence to read:

Finally resuspend the cells in 10 cc. of sterile suspension medium, add 1 cc. of this suspension to 10 cc. of sterile suspension medium, and mix.

Official and Released February 20, 1952

By authority of the U.S.P. Board of Trustees and of the Executive Committee of Revision.

ROBERT L. SWAIN, *Chairman*
U.S.P. Board of Trustees

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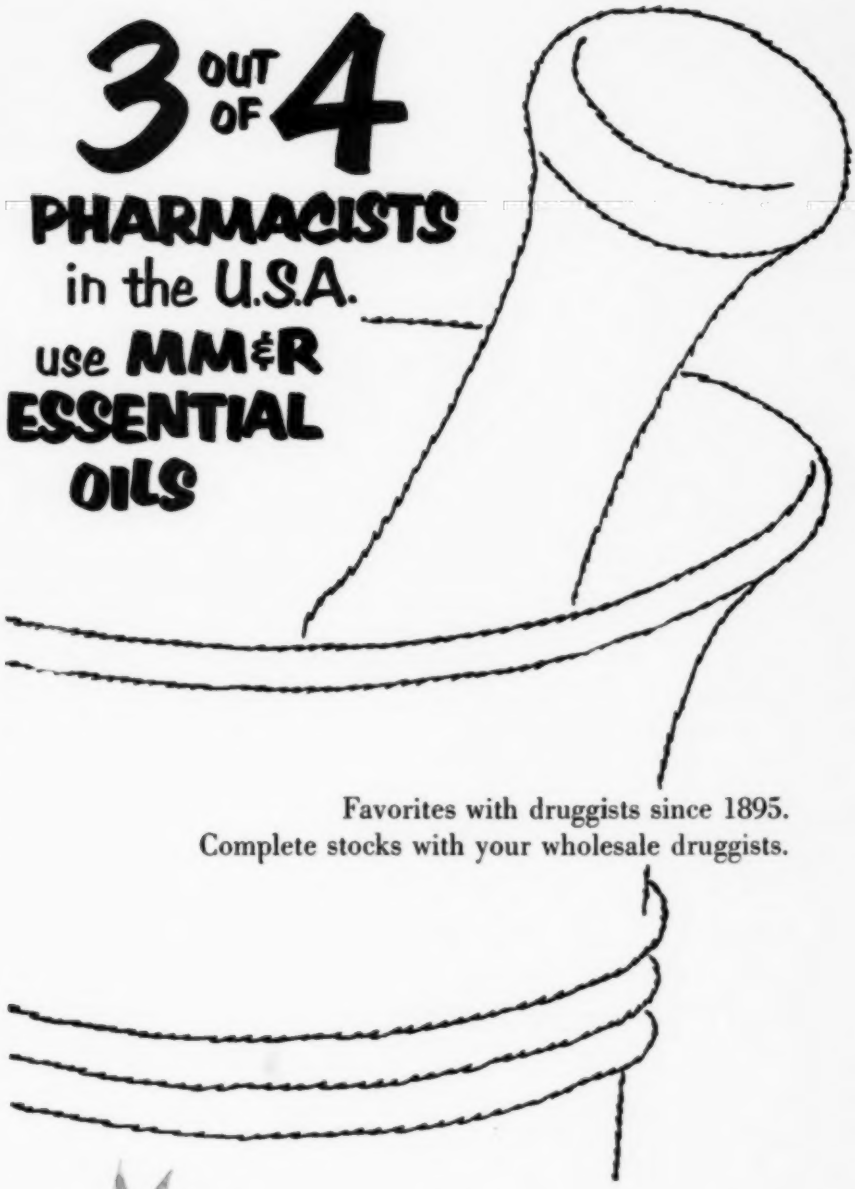

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